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14. ABSTRACT
Rheumatoid arthritis (RA) and multiple sclerosis (MS) are chronic inflammatory autoimmune diseases affecting millions of people. Here we are proposing a novel approach to cure MS, by administration of a specific strain of human commensal-bacteria. Recent studies have shown that intestinal microflora plays an important role in the health of the host and posses probiotics like qualities. We hypothesize that Gram-negative commensal bacteria from human gut have the potential to be used as a therapeutic agent. We have used collagen induced arthritis (CIA) in HLA-DR4DQ8 mice and PLP91-110 induced experimental autoimmune encephalomyelitis (EAE) HLA-DR3DQ8 mice to test our hypothesis that treatment with commensal bacteria Prevotella histicola can modulate disease. First using various doses of bacteria, we have identified the optimal dose to be used for treatment of CIA as well as EAE. Our in vitro and in vivo data showing suppression of antigen-specific immune response in EAE and arthritis in P. histicola treated mice suggest generation of peripheral tolerance via gut. Our studies show that treatment with P histicola suppresses arthritis/EAE via gut and generation of regulatory DCs and T regulatory cells. Our data indicates that P histicola induced immune responses in the gut cause induction of immune tolerance in periphery leading to suppression of antigen specific response.

15. SUBJECT TERMS
Commensal, inflammatory disease, therapeutic agent, multiple sclerosis, rheumatoid arthritis, HLA transgenic mice, tolerance, P histicola.

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This progress report is from Feb '2011 to April '2012

Introduction

Rheumatoid arthritis (RA) and multiple sclerosis (MS) are chronic inflammatory autoimmune diseases affecting millions of people. Since these diseases occur more often among young and middle-aged adults, they cause significant loss of productive years in this population. Beside these autoimmune diseases also cause significant economic burden (hundreds of Billions of USD) on society. Although several therapies are in use, none of them cure disease. In this study we are investigating a unique approach to ameliorate RA and MS, by administration of a specific strain of human commensal bacteria *Prevotella histicola* (*P histicola*), which was recently isolated from human gut. Among all the genetic factors linked with RA and MS, the strongest association has been with the MHC class II region on chromosome 6 (1) and we have generated novel humanized HLA class II transgenic animal models of RA and MS. We are utilizing these animal models to test the therapeutic efficacy of *P histicola*. Using an experimental autoimmune encephalitomyelitis (EAE), which is an animal model of MS, we showed that HLA-DR3DQ8 transgenic mice develop MS like disease characterized by brain plaques (2). HLA-DR3DQ8 mice were immunized with CNS antigen PLP91-110 and received either *Prevotella histicola* or medium staring day 7 post-immunization and every other day for a total of 7 doses. Previously we have shown that treatment of mice with 3-4 doses of *P. histicola* in PLP-immunized mice i) led to suppression of antigen-specific immune response *in vitro*; ii) suppressed inflammatory cytokine IL-17 and increase in levels of anti-inflammatory cytokine IL-10; and iii) lower clinical disease incidence as well as severity indicating immunosuppressive properties of *P histicola*.

In 2nd year we have observed that P histocola treatment can suppress ongoing EAE in DR3DQ8.AEo transgenic mice. Treatment of DR3DQ8 mice with EAE on day 11 with *P histicola* suppressed or down regulated disease severity in 50% of mice. Next we investigated requirement of live vs. dead bacteria for therapeutic effect and observed that live bacteria is needed for suppressing EAE as heat killed bacteria failed to suppress EAE in DR3DQ8 transgenic mice significantly. We also performed histopathology of brain as well as spinal cord and have observe that *P histocola* treated group have reduced brain and spinal cord pathology compared to medium treated group. Treated group also showed reduced number of inflammatory cells in CNS as well as reduced level of inflammatory cytokine such as IFNγ and IL-17. Further we have standardized method to isolate CD4 regulatory T cells and suppressive macrophages. Our preliminary studies show that P histicola mediate its immunosuppressive activity through increase in regulatory CD4 T cells and tolerogenic CD103+CD11c+ dendritic cells (DCs).

Similarly, we studied therapeutic efficacy of *P histicola* to modulate arthritis in murine model of RA known as collagen induced arthritis (CIA) (3). Our in vitro studies showed that treatment of mice with *P. histicola* in collagen-immunized mice led to suppression of antigen-specific immune response and reduction in production of inflammatory cytokines. In the second year we show that P. histicola can protect DQ8 and DR4/DQ8 mice from arthritis in protective and therapeutic protocol. On the other hand, treatment with a control bacteria Prevotella melanogenics did not result in protection from arthritis. We further show that arthritis is regulated via generation of T regulatory cells and regulatory DCs. The treatment led to increased production of regulatory cytokines like IL-10 with lower levels of IL-17. Further, rtPCR for various cytokines and chemokines showed that treatment led to lower expression of pro-inflammatory IL-23. To determine if gut immune system is different in naïve mice we used DRB1*0401 mice that show sex-biased arthritis and also *0402 mice that are resistant to arthritis (6). Our data shows that gut immune system is different between male and female *0401 mice and also between *0401 and *0402 mice suggesting a crucial role of gut in pathogenesis (7, 8). This also confirms our hypothesis that arthritis can be modulated via gut and such a therapy may be possible in humans. Thus in summary, we have made good progress towards completing our aims propsed in the grant and our data suggests that *P histicola* induced immune responses in the gut might cause induction of immune tolerance in periphery leading to suppression of antigen specific response.

Progress report (arranged according to approved SOW)

SOW 1-e) Immunization of mice with relevant antigen and feeding bacteria in ongoing disease in vivo (11-12th months)

CIA 20 mice Controls 20 Dr. Taneja **Completed**

EAE 20 mice Controls 20 Dr. Mangalam **Completed**

SOW 1-f) Score immunized mice for arthritis in CIA model. Bleed mice via tail technique (11-16th months) **Dr. Taneja**.

Mice were immunized with type II collagen (CII) to induce arthritis and the fed them P histicola on alternate days 2 weeks following immunization. Next we tested mice in preventive protocol. Mice were fed P histicola 12 days prior to immunization with type II collagen. Mice immunized with type II collagen and fed media without bacteria as well as mice fed bacteria without CII immunization were used as controls. Disease phenotype in CIA model is characterized by paw swelling, scored on scale of 0-3. Our data showed that both preventive and therapeutic protocol suppressed disease incidence and antibodies to Collagen II. We used a control bacteria P. melanogenica; it did not show any suppressive activity (Fig.1).

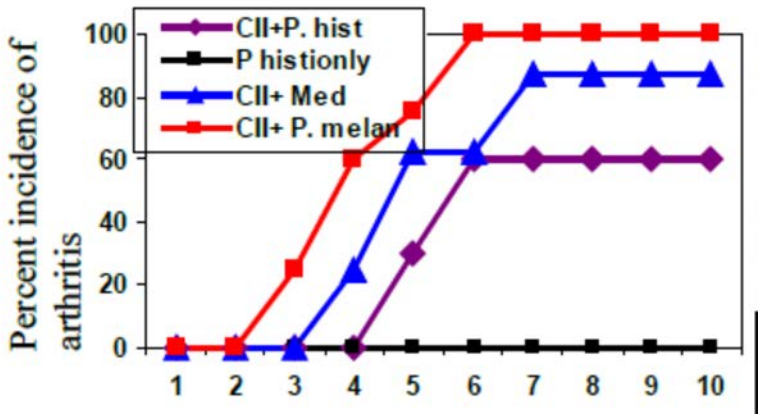

Fig 1. Treatment with P. histicola of mice induced for collagen-induced arthritis suppresses disease incidence while a control bacteria P. melanogenica did not show suppressive activity. P. histicola alone did not lead to arthritis.

Score immunized mice for paralysis in EAE model. Bleed mice via tail technique (11-16th months) Dr. Mangalam

In these set of experiments, we wanted to investigate if *P histicola* can suppress or modulate an ongoing disease in EAE or CIA model. As shown in Tab 1, P histicola treatment of DR3DQ8.AEo mice with EAE lead to 50% decrease in disease incidence and severity as compared to medium treated control group, which showed 100% disease incidence. P histicola treated group also showed less severe disease as compared to control group as shown be cumulative clinical score in Fig. Medium treated control group with EAE showed a cumulative score of 83 [9 mice with score of 5 (45), 5 mice with score of 4 (20), and 6 mice with a score of 3 (9)], while P histicola treated mice had a cumulative score of 28 [1 mice with score of 5 (5), 2 mice with score of 4 (4), 3 mice with a score of 3 (6), 4 mice with a score of 2 (2) and 10 mice with no disease (0)]. Thus our data indicate that P histicola can modulate an ongoing disease.

Table 1: Effect of P histocola on PLP91-110 induced EAE in DR3DQ8.AEo transgenic mice

Mouse strain	Disease incidence (%)	Mean onset of disease ± SD	Number of mice with maximum severity score				
			1	2	3	4	5
DR3.DQ8.AE^o (Medium fed)	20/20 (100%)	10.5±1	-	-	4	10	6
DR3.DQ8.AE^o (*P*	10/20	15±2	-	4	3	2	1

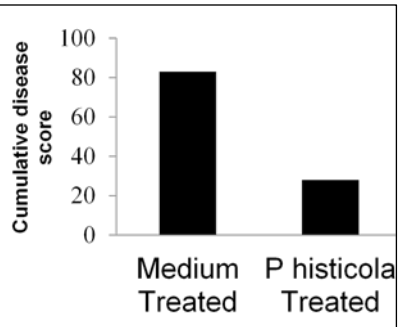

Fig.2. P histicola treated mice show decreased cumulative clinical score as compared to sham treated mice in an ongoing EAE model

***histicola*)**	**(50%)**						

Feeding bacteria in ongoing disease modulate disease severity and treated group showed decrease in disease severity compared to control.

SOW 1-g) Sacrifice CIA group mice and harvest various organs and snap freeze a part of organs and one part for paraffin blocks (16-17th months) Dr. Taneja
Completed

Sacrifice EAE mice and harvest various organs and snap freeze a part of organs and one part for paraffin blocks (16-17th months) Dr. Mangalam
Completed

SOW 1-h) Harvest spleen and lymph nodes from the treated and controls mice in CIA (arthritis) model and do *in vitro* assay for T cell response to antigen and measure cytokines (17 month) Dr. Taneja
CIA (arthritis) model (20th month). Dr. Taneja (completed last year)

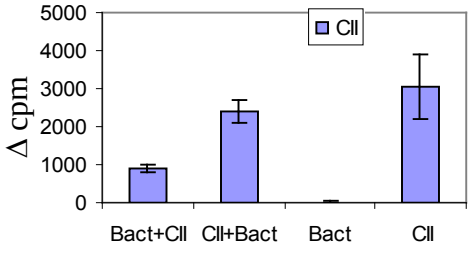

Fig 3. Lymph node T cell response to type II collagen in DR4/DQ8 transgenic mice fed P histicola before and after CII-immunization and control CII-immunized without bacteria and only bacteria fed mice.

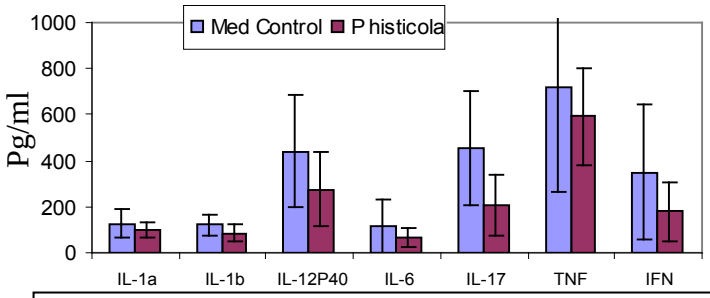

Fig 4. Cytokine production analyzed by ELISA in supernatants of mice immunized with CII and fed media (Med Control) or P histicola. Transgenic mice fed P histicola show a much lower production of proinflammatory cytokines.

Prevotella histicola modulates antigens-specific responses: Since collagen specific T-cell responses play an important role in disease pathogenesis of CIA, we investigated effect of *Prevotella* on antigen specific immune response and production of pro-inflammatory cytokines by antigen specific T-cells. Mice were fed bacteria before or after immunization with CII. Mice immunized with but no bacteria and mice fed bacteria without CII-immunization were used as controls. As shown below in Fig 3, antigen-specific T cell response was suppressed in mice fed P. histicola before and after immunization with CII as compared to mice immunized with CII only. As expected, mice fed bacteria in the absence of CII-immunization did not show any antigen specific response. We further tested and compared production of pro-inflammatory cytokines in mice immunized with CII and fed medium and mice immunized with CII and fed *P histicola* (Fig 4). Mice treated with bacteria after CII-immunization showed a much lower production of proinflammatory Th1 (IL-1, TNF and IFN) as well as Th17 (IL-12(p40), IL-17, IL-6) cytokines compared to mice immunized with CII and fed media without bacteria. These in vitro studies clearly show an immunomodulatory role of commensal bacteria like P histicola. Our studies suggest that *P. histicola* may be able to generate systemic suppression via mucosal immune regulation.**SOW 1-h**) Harvest spleen and lymph nodes from the treated and controls mice in EAE (multiple sclerosis) model and do *in vitro* assay for T cell response to antigen and measure cytokines (17 month) Dr. Mangalam

EAE (MS) model (20th month). Dr. Mangalam (completed last year)

Modulation of antigen specific T cell and cytokine response by *P histicola*

To determine if this protective effect of *P. histicola* is due to own-regulation of antigen specific T cell responses, we isolated splenocytes from mice treated with bacteria or medium and stimulated with PLP_{91-110} peptide. As shown in Fig. 5, antigen specific T cell response was suppressed in DR3DQ8 mice treated *P. histicola* as compared to sham treated mice. Splenocytes from bacteria fed mice also produce less pro-inflammatory cytokine IL-17 on stimulation with PLP, while levels of anti-inflammatory cytokine IL-10 was increased (Fig. 6). Levels of IFN-γ were not significantly different between two groups (Fig. 6).

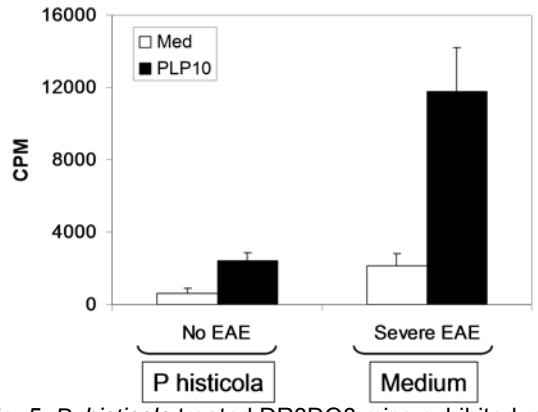

Fig. 5. *P. histicola* treated DR3DQ8 mice exhibited reduced PLP_{91-110} specific T cell proliferation as compared to sham treated mice. Splenocytes were collected from mice immunized with PLP and treated with *P. histicola* or medium (sham) and were stimulated *in vitro* with the PLP_{91-110} polypeptide.

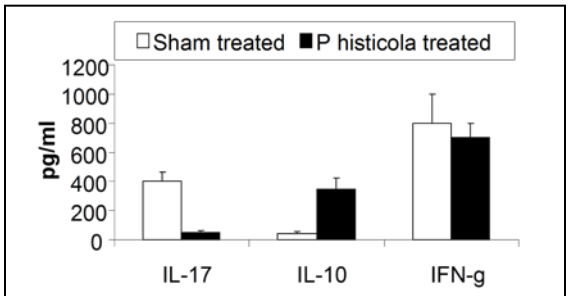

Fig.6 - *P. histicola* treated DR3DQ8 mice exhibited reduced level of IL-17 and increased levels of IL-10 as compared to sham treated mice. Levels of IFN-γ were not different

Milestone#7 immunomodulatory effect of bacteria in disease.

1-i) Sections of paraffin blocks and frozen blocks from CIA (arthritis) group and staining with heamatoxylin and eosin. ELISA for antibodies(18-20 months) Dr. Taneja

To ensure that treatment of mice did not result in any pathology of the gut, we did histopathology of mice immunized with CII and treated with either medium or with P histicola. We observed that P. histicola treatment did not cause any pathology in the gut (Fig.7).

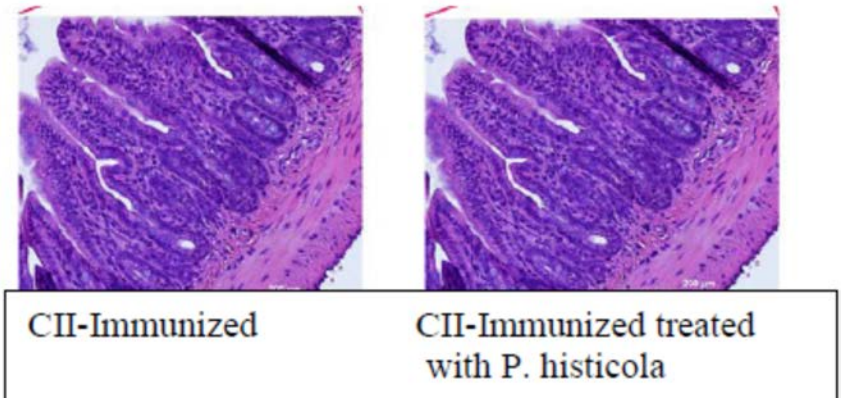

Fig.7. H&E stain of gut of mice treated either with P histicola or left untreated in CIA model.

Sections of paraffin blocks and frozen blocks from EAE (multiple sclerosis) group and staining with heamatoxylin and eosin. ELISA for antibodies(18-20 months**) Dr. Mangalam**

Immunization of DR3DQ8 transgenic mice with PLP antigen lead to development of EAE. The disease is characterized by inflammation and demyelination in spinal cord as well as brain tissue, resulting in neurological deficit. Although, treatment with P histicola suppressed clinical disease, it was still possible that treated mice have brain and spinal cord pathology. Therefore we analyzed these tissues and observed that P histocola treated group showed decreased inflammation and demyelination compared to sham treated group (Fig 8). Bacteria treated group also showed decreased levels of inflammatory cells (Fig 9A), as well as inflammatory cytokines (IL-17 and IFNγ) (Fig 9 B and C) compared to medium treated group. Treated group also showed decreased levels of anti-myelin antibodies (data not shown).

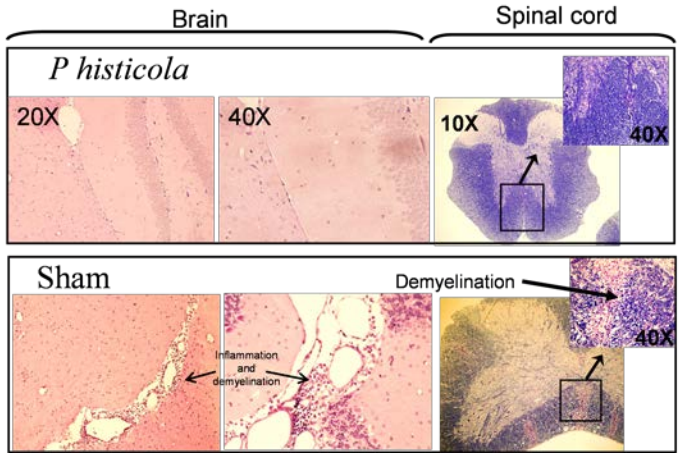

Fig. 8. P histicola treated group showed decreased inflammation and demylination in brain as well as spinal cord compared to control untreated group.

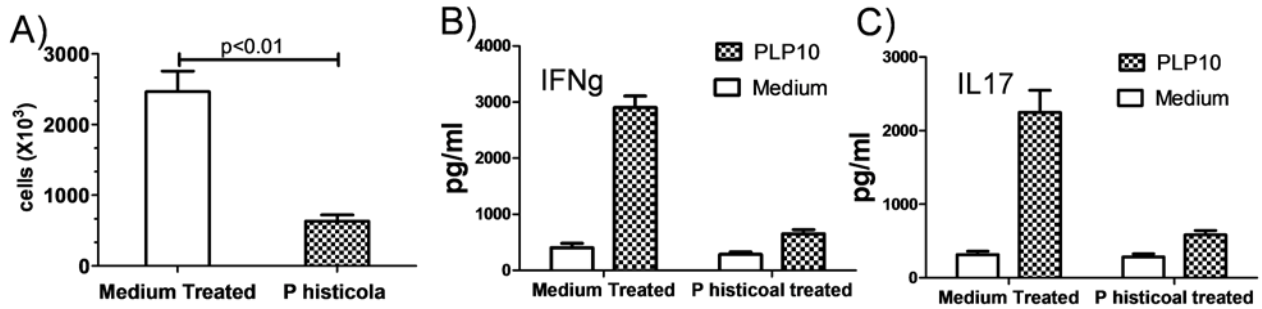

Fig. A) P histicola treatment reduces brain infiltrating cells in PLP91-110 immunized mice compared to medium treated mice. P histocola treated mice also show reduced levels of inflammatory cytokine IFNγ and IL-17 confirming its immunomodulatory role in EAE

Milestone# 8. Histopathology of various organs and antibodies for diagnosis of disease**.**

1-j) compilation of in vivo data and T cell response and cytokine data in CIA (arthritis) model (20th month). Dr. Taneja

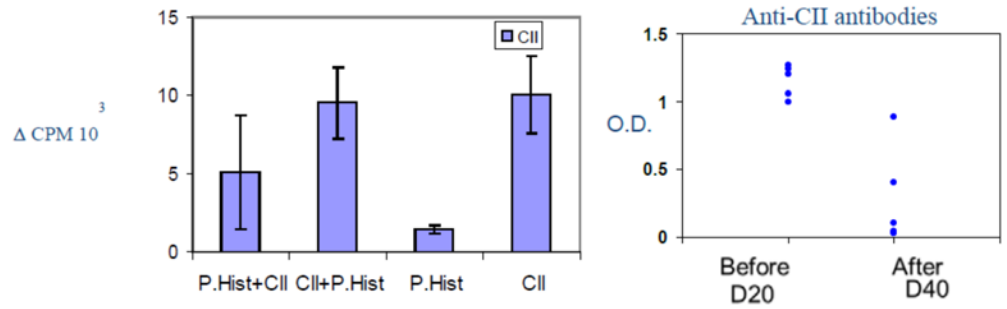

Fig 10. DQ8 mice were immunized with type II collagen (CII) and treated with P. histicola showed suppression of T cell response when treatment was before immunization. Autoantibodies to CII were significantly reduced in P. histicola treated mice. Sera collected at day 20 after immunization with CII, and day 40 after treatment started at day 21 were tested by ELISA.

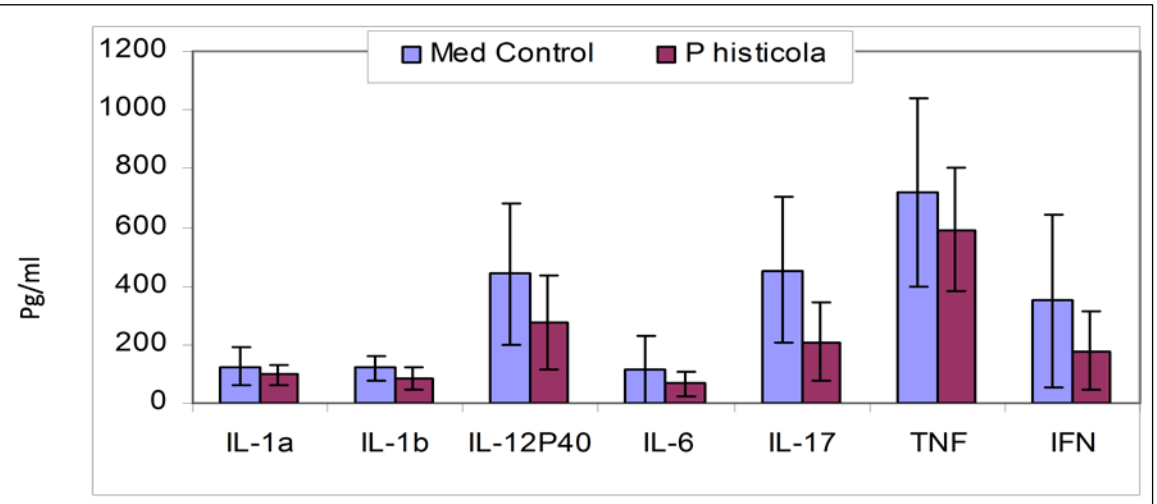

Fig 11. Cytokines in medium treated and P. histicola treated mice showed suppression of proinflammatory cytokines.

In vivo data is compiled in Fig 11. T cell response was significantly decreased when mice were first treated and then immunized with CII compared to untreated mice. However, treatment after CII immunization did not significantly reduce T cell response suggesting regulation of disease could be via other regulatory mechanism.

Compilation of in vivo data and T cell response and cytokine data in EAE (multiple sclerosis) model (20th month). Dr. Mangalam

Analysis of T cell response and cytokine in in vivo study, showed that treatment with P histicola led to suppression of antigen response only in lamina propia, while T cell response in mesenteric lymph node and spleen were not affected (12A). However, P histicola treated and protected mice showed decreased levels of pro-inflammatory cytokines (12B).

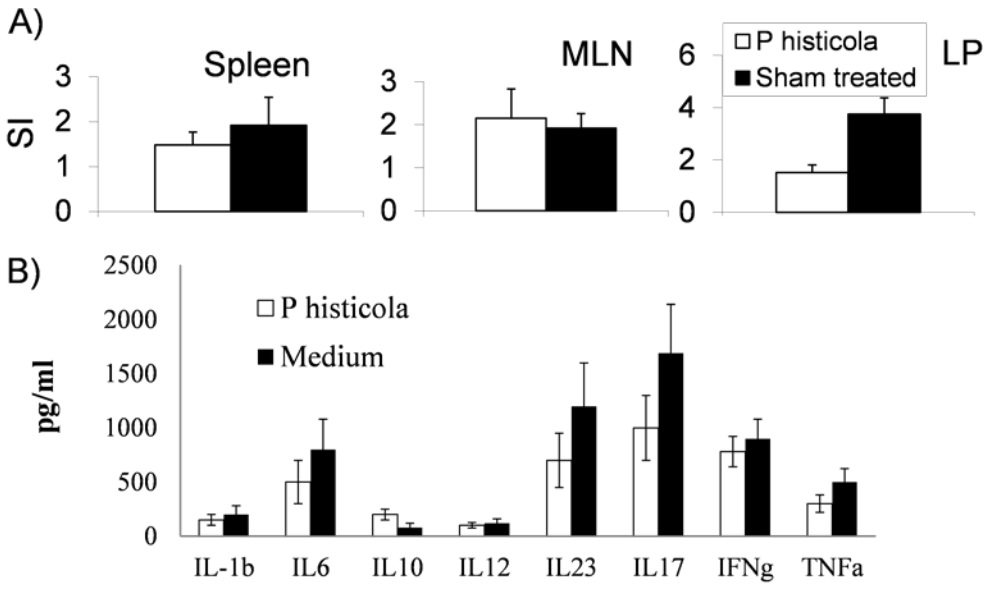

Fig 12.A) P histicola treatment suppressed antigen specific response only in Lamina propia (LP) cells but not in Mesenteric Lymph node (MLN) cells or spleen. B) Cytokines in medium treated and P. histicola treated mice showed suppression of proinflammatory cytokines.

Milestone#9 Analysis of data and publication (2 publications expected one for each disease).
Four abstracts, two in April'2011, one in Nov'2011 and Mar 2012, one manuscript 2012.

Aim#2 Mechanism of anti-inflammatory action of commensal bacteria *P histicola.*

2a) Heat inactivate bacteria and feed in therapeutic protocol and monitore for disease (18-24 months)
Mice sacrificed CIA 20 Controls 20 Dr. Taneja
Experiments with EAE model did not show any difference in heat killed and live bacteria. Considering this, this experiment was not carried out for CIA studies as while EAE model can be done in one month, CIA takes 4-6 month to complete. It was decided not to waste valuable mice and manpower.
EAE 20 Controls 20 Dr. Mangalam **Ongoing** (done once being repeated

Table 2: Requirement of Live vs. heat killed P histocola on PLP91-110 induced EAE in DR3DQ8.AEo transgenic mice

Mouse strain	Disease incidence (%)	Mean onset of disease ± SD	Number of mice with maximum severity score				
			1	2	3	4	5
DR3.DQ8.AE° (Medium fed)	10/20 (100%)	10.5±1	-	-	4	2	4
DR3.DQ8.AE° (*P histocola live*)	2/10 (20%)	15±2	-	1	-	-	1
DR3.DQ8.AE° (*P histocola heat killed*)	8/10 (80%)	14±2	-	1	1	2	4

Milestone# 10 Our preliminary data indicate that heat killed bacteria do not modulate disease suggesting that live bacteria is required for disease suppression.

2b) Feed mice bacteria for a week and then immunize with Type II collagen for DR4/DQ8 and PLP for DR3/DQ8 mice**.** Harvest LNCs and spleen cells duodenum, ileum, jejunum and colon after 10 days of immunization. (21-22 months)
DR4/DQ8 mice -2 Dr. Taneja
DR3/DQ8 mice -2 Dr. Mangalam

We have stardaized this method and observed that we need 5 mice to get enough cells to perform mechanistic experiments.
2c) Repeat experiment in 2a and
isolate cells from mice and pool cells from mice necessary for T cell response. Characterize cells, T reg, memory T, DCs, Mac, B cells and TLR expression in various parts of intestines, T cell responses, freeze supernatants from culture (22-24 months)
DR4/DQ8 mice- 10 mice Controls 10 Dr. Taneja

2b, 2f, 2g) Characterization of T regulatory cels from intestine and periphery and cytokines. *Prevotella histicola* modulates antigen-specific responses via generation of T regulatory and regulatory DCs. These studies suggested *P. histicola* may be able to generate systemic suppression via mucosal immune regulation. We tested various cells in CII-immunized and mice immunized and treated with bacteria in spleen and lamina propria. As shown in Fig 13, both spleen and lamina propria showed an increase in T regulatory, CD4+CD25+FoXP3 and CD4+GITR+, cells as well as suppressive DCs that express CD103. These studies suggest that treatment with P histicola leads to generation of regulatory DCs that activates more T regulatory cells, these T cells and DCs can produce regulatory and anti-inflammatory cytokines. Both T reg and reg DCs can migrate into the systemic immune system.

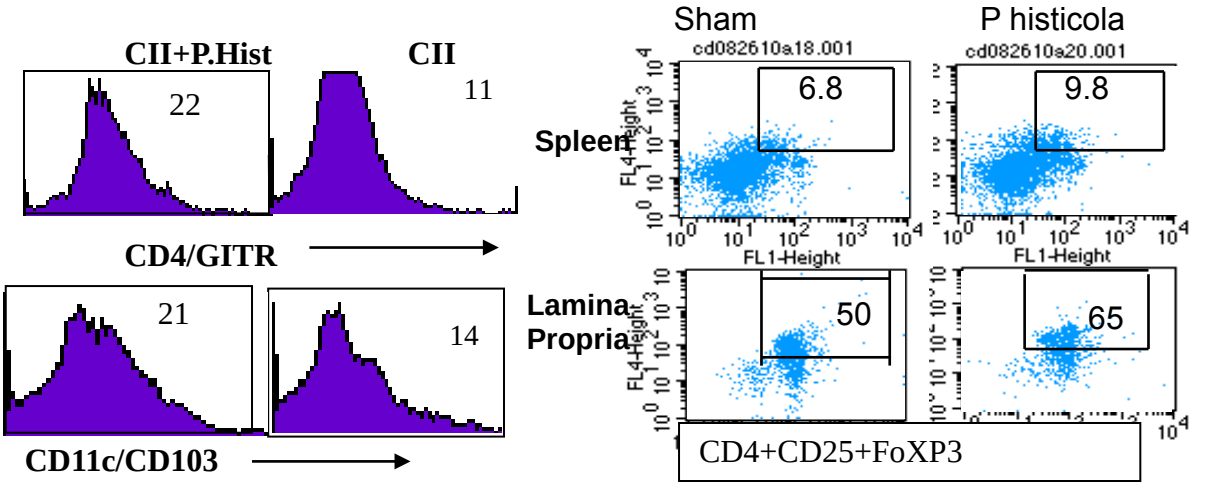

Fig 13. Treatment of DQ8 mice with P.histicola resulted in generation of regulatory DCs and T regulatory cells as observed in splenic and lamina propria isolated cells. Various cells were enumerated by FACS analysis.

***Prevotella histicola* regulates systemic immune response via DCs and production of IL-10.**
Mice treated with *P. histicola* and immunized with CII had DCs that when cultured with CD4 cells from treated or Sham mice suppressed immune response when challenged in vitro with CII. Supernatants from these cultures showed that treated DCs and CD4 led to production of IL-10 while IL-17,

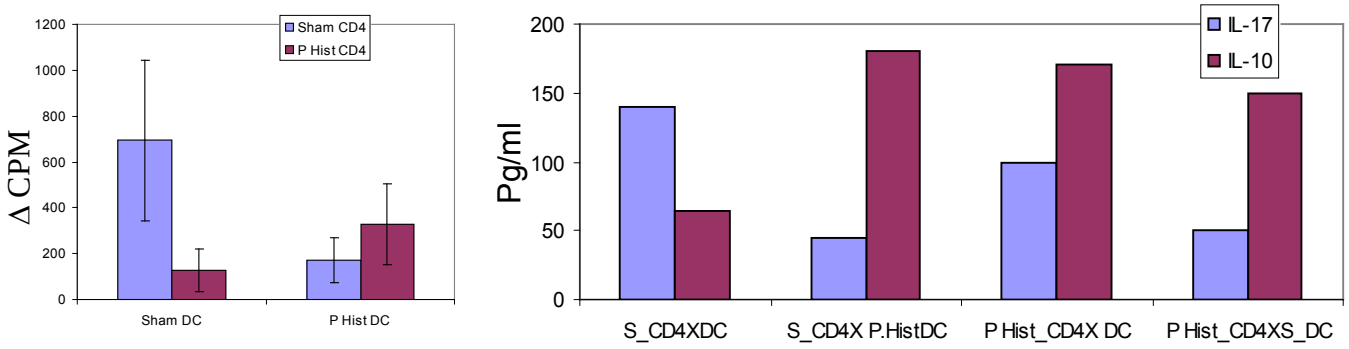

Fig 14. P. histicola suppresses systemic immune response via dendritic cells. Mice treated with P. histicola or medium (Sham) were immunized with CII. Dendritic cells (DCs) and CD4 T cells were isolated from spleen and in vitro challenged with CII in a criss-cross culture. As shown in right panel, DCs from treated mice led to suppression of CD4+ T cell response in vitro. Also, when DCs and CD4+ cells of treated mice were cultured, there was increased production of IL-10 (right panel).

proinflammatory cytokine, was suppressed in comparison to sham treated mice that produced more IL-17 and lower IL-10. These studies suggest that *P. histicola* treatment has led to generation of regulatory DCs phenotypically and functionally (Fig 13 and 14).

DR3/DQ8 mice-10 mice Controls 10 Dr. Mangalam

P histicola can modulate disease by inducing anti-inflammatory immune response mediated by regulatory cells such as CD4+FoxP3+ Tregs, suppressive macrophage and or tolerogenci DCs. To determine if these cell subsets are involved in the suppressive effect of Phisticola, We isolated Tregs, macrophage and DCs from P histicola treated and PBS treated mice. Our data indicate that P histicola modulate disease through increase in Regulatory T cells number (Fig. 15 A and B) as well as their function. CD4+CD25+ Tregs isolated from P histicola treated mice showed increased ability to suppress antigen specific immune response as compared to medium treated control group (Fig 15 C). Treatment with P histicola also resulted in increase in number of tolerogenic DCs (Fig. 16 A), which have reduced antigen presentaion capacity as compared to DCs isolated from naive mice or medium treated control group (Fig 16B). Tolerogenic Dcs are characterized by high IL-10 to IL23 ratio and we also observed a similar phenotype as DCs isolated from bacteria treated group produce more IL-10 than IL23 (Fig16 C and D). Thus P histicola suppress disease throgh modulation of Tregs, and tolerogenic DCs, which lead to decreased antigen presentation and inflammation.

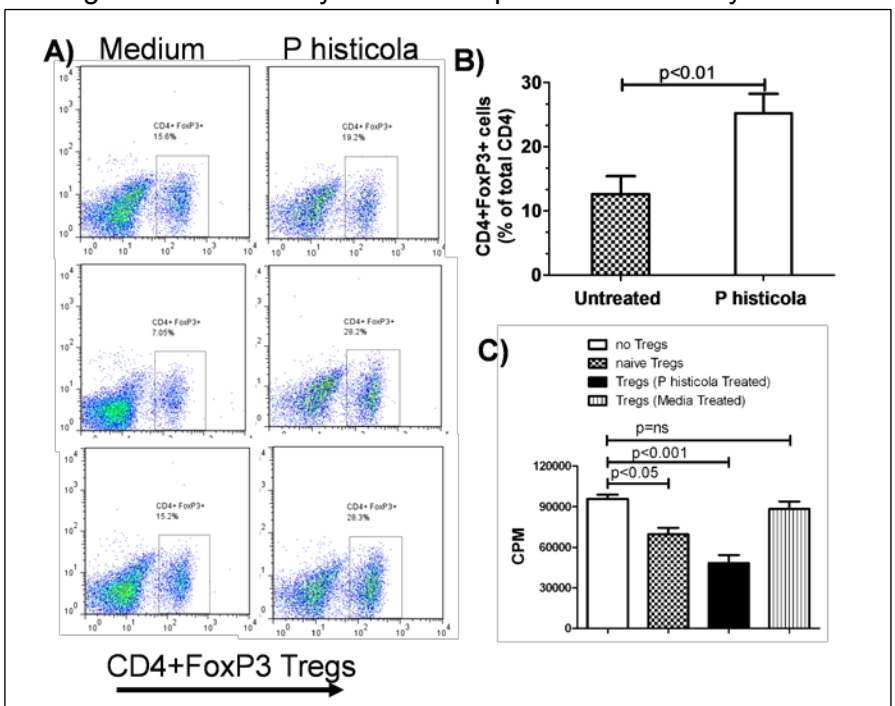

Fig. 15 A) P histcola treated mice show increase in CD4+FoxP3+ regulatory T cells compared to o medium treated group. B) histogramplot for Tregs in treated vs untreated group. C) CD4+CD25+ Tregs from P histocola treated mice have increased suppressive activity as compared to medium treated group.

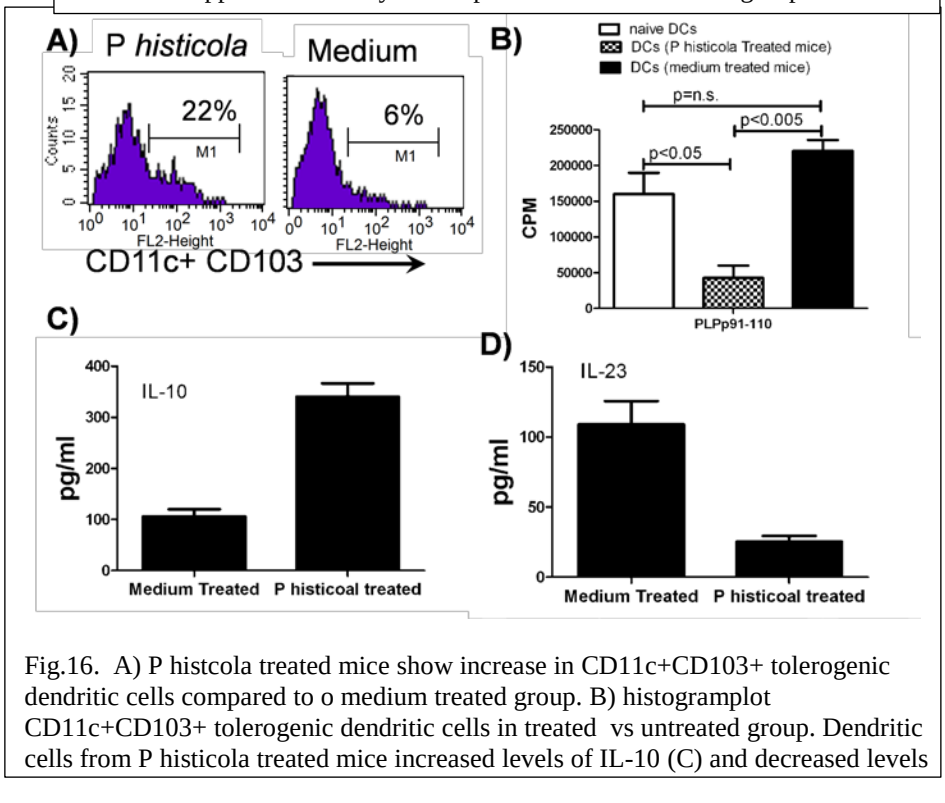

Fig.16. A) P histcola treated mice show increase in CD11c+CD103+ tolerogenic dendritic cells compared to o medium treated group. B) histogramplot CD11c+CD103+ tolerogenic dendritic cells in treated vs untreated group. Dendritic cells from P histicola treated mice increased levels of IL-10 (C) and decreased levels

Milestone#11
Immunomodulatory effects of bacteria in immune response.
2d) Analysis of the data generated by heat killed bacteria and cytoplasmic component and in vitro data DR4/DQ8 mice Dr. Taneja **Ongoing**
DR3/DQ8 mice Dr. Mangalam Ongoing

2g, h and i) Cytokines, primer design and RT-PCR in gut

Expression of cytokines/chemokines in CIA (arthritis) model Dr. Taneja

Next we determined the effect of treatment in jejenum of DQ8 mice and compared to control by doing rtPCR for various cytokines. In addition, we also used naïve *0401 and *0402 mice to determine if gut immune system can impact susceptibility in association with genotype.

rtPCR for cytokines in jejenum: We did rtPCR for various cytokines in jejenum of mice treated and immunized with CII and those only immunized with CII using published primers. As shown in Fig 17, mice treated with *P.histicola* showed a much higher expression of anti-inflammatory cytokines, IL-4 and IL-10 and lower expression pro-inflammatory cytokine IL-23 further confirming that treatment modulates arthritis via gut As shown above, *P.histicola* alone did not cause arthritis or any pathology suggesting, this might be a good treatment for clinical trials in RA patients.

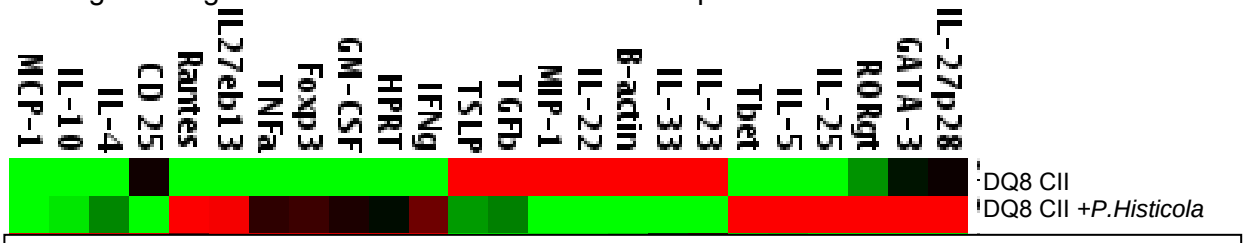

Fig 17 rtPCR for cytokines in jejenum of DQ8 mice immunized with CII and treated with P. histicola compared to CII immunized mice showed differences in pro and anti-inflammatory cytokines.

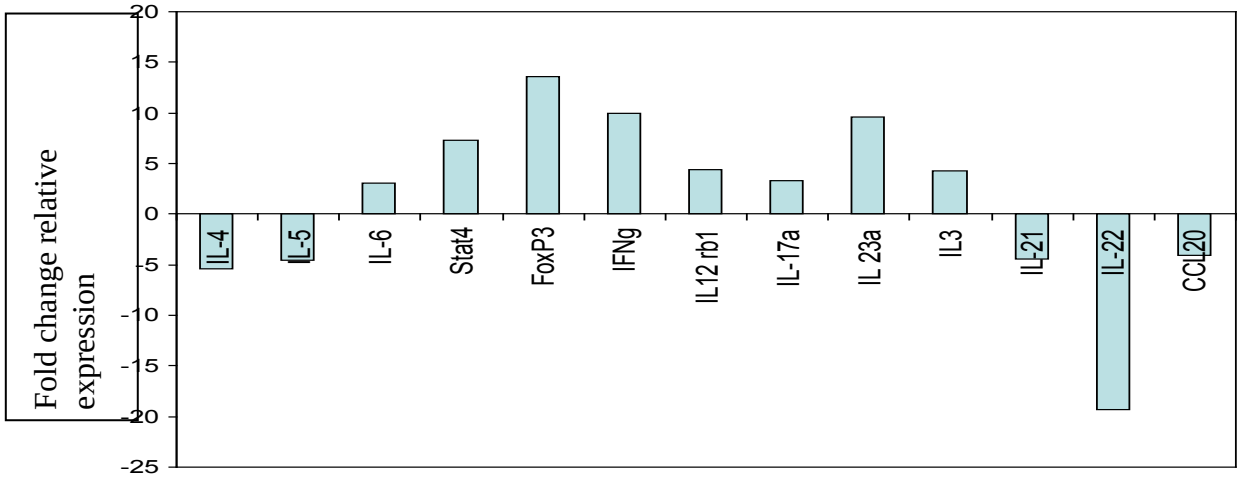

Fig 18 Comparison of fold change in gene transcript levels between *0401 females and males.

***0401 female mice show different jejenum profile than males.**

We tested the jejuna of naïve mice for expression of cytokine and chemokine transcripts involved in the Th17 regulatory network by rtPCR (Figure 18). Susceptible *0401 females showed a distinct cytokine and chemokine profile as compared to males that was characterized with a significant increase in IL-23α and IFNγ along with a decrease in the regulatory cytokines IL-4, IL-22 and CCL20. Similarly, *0401 females showed more than 3 fold increased gene transcripts for Th17 cytokines IL-17, IL-23, IL-6 and Th1 cytokines IFNγ, Stat 4 and TBX21 while *0402 females had several fold increase in genes regulating Th2 cytokines and regulatory networks like ICOS, GATA3 and IL-4. *0401 male mice did not show an increase in transcripts for TH17 encoding genes compared to *0402 mice.

Our data showed a bias towards TH1/TH17 cytokine expression with significant decrease in cytokine gene transcripts required for negative regulation of Th17 profile, like IL-4, IL-21 and IL-22, in *0401 females as compared to *0401 males and *0402 females. Interestingly CCL20 and CCL22 which are required for the generation of regulatory CD4 T cells and DCs, are reduced several fold in *0401 females as compared to *0401 males and *0402 females. These data suggest that events in gut may be involved in pathogenesis.

Expression of cytokines/chemokines in EAE (multiple sclerosis) model Dr. Mangalam

To determine the effect of *P histicola* on mucosal immune system, duodenum, jejunum and ileum were isolated from P histicola treated and medium treated mice immunized for EAE.

RNA was extracted from tissue, reverse transcribed into cDNA and expression of cytokines and transcription associated with anti-inflammatory response were analyzed by Real time PCR using specific primers. As shown in figure 18, P histicola treated mice showed increased levels of anti-inflammatory cytokine such as TGFβ, IL-10, IL-25 and TSLP compared to control group with EAE. The levels differed between tissue but maximum expression was observed in ileum. Bacteria treated group also showed increase in levels of FoxP3, a transcription factors marker for the regulatory CD4 T cells.

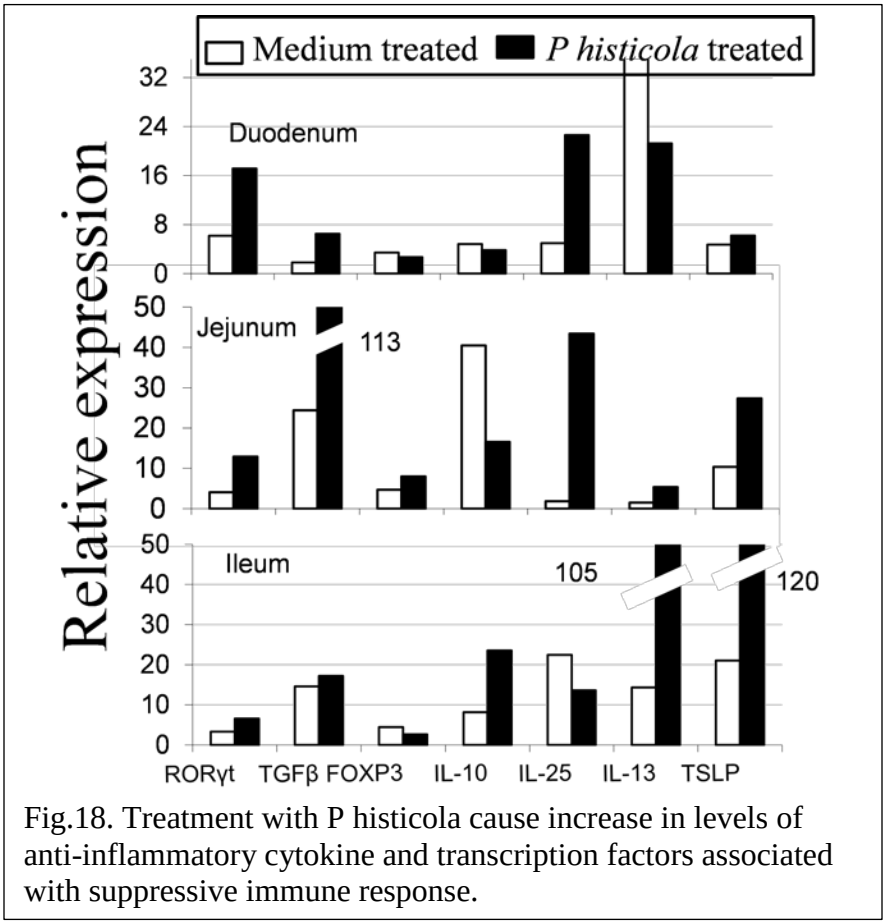

Fig.18. Treatment with P histicola cause increase in levels of anti-inflammatory cytokine and transcription factors associated with suppressive immune response.

These data suggests that treatment with *P histicola* cause increase in levels of Th2 and anti-inflammatory cytokines. These cytokine might be responsible for modulation of inflammatory immune response in periphery leading to down-regulation of pro-inflammatory response and disease.

Milestone#13-15 cytokines, design of primers for mRNA expression for cytokine/chemokine expression in intestines.

The Key Research Accomplishments

1st Year

- Culture of P Histicola for use in both CIA and EAE model

- Generation of DR4/DQ8 transgenic mice for in vivo use. HLA-DR4 and HLA-DQ8 transgenic mice are mated to generate double transgenic mice. Double transgenic mice are characterized for the presence of HLA transgenes by flow cytometry using specific conjugated antibodies. Mice positive for both genes are identified and mated. DR4 and DQ8 transgenes can segregate which necessitates typing for the transgene positivity.

- Generation of DR3/DQ8 transgenic mice for in vivo use. HLA-DR3 and HLA-DQ8 transgenic mice were mated to generate double transgenic mice. Double transgenic mice are characterized for the presence of HLA- DR3 and –DQ8 transgenes by flow cytometry using specific conjugated antibodies. Mice positive for both genes are identified and mated. DR3 and DQ8 transgenes can segregate which necessitates typing for the transgene positivity.

- Mice were gavaged with P histicola for 2 weeks and then immunized with either type II collagen (DR4DQ8 mice) PLP_{91-110} peptide (DR3DQ8 mice). In addition, control mice were gavaged with media in which *P histicola* were cultured and immunized with type II collagen or PLP_{91-110} peptide. Mice are being monitored for disease (CIA in DR4DQ8 and EAE in DR3DQ8).

- Sera from all test and control mice is being collected and will be used to study antibodies at the termination of the experiment.

- In vitro experiments show that feeding bacteria suppressed antigen-specific T cell response and reduced production of inflammatory cytokines in both CIA and EAE models.

2nd Year

- Mice were gavaged with P histicola for 2 weeks and then immunized with either type II collagen (DR4DQ8 mice) PLP_{91-110} peptide (DR3DQ8 mice). In addition, control mice were gavaged with media in which *P histicola* were cultured and immunized with type II collagen or PLP_{91-110} peptide. Treatment with P histocola suppressed disease incidence and severity in both CIA as well as EAE model.
- In vitro experiments show that feeding bacteria suppressed antigen-specific T cell response and reduced production of inflammatory cytokines in both CIA and EAE models.

Milestone#8. P histocola treated group showed decreased inflammation and demyelination compared to sham treated group. Treated mice also showed decrease levels of inflammatory cytokine IL-17 and IFNγ. Histopathology of in vivo studies and antibodies in mice treated with P. histicola have been completed. • Treatment with P histocola suppressed RF and anticitrullin antibody in CIA model. Treatment with P histocola suppressed anti-myelin antibody in EAE model.

Milestone #9 Data has been presentation at National meetings and manuscript is being analyzed for publication.

Milestone# 10. Our preliminary data indicate that heat killed bacteria do not modulate disease suggesting that live bacteria is required for disease suppression. We have performed this experiment once and repeating it one more time to confirm the finding.

We have also standardized isolation of cells from various organs

Milestone#11 Immunomodulatory effects of bacteria in immune response. We have isolated Tregs, macrophage and DCs from *P histicola* treated and PBS treated mice. Our data indicate that *P histicola* modulate disease through increase in Regulatory cells number as well as suppressive function of Tregs. Treatment with *P histicola* also leads to generation of tolerogenic DCs and macrophages which have reduced antigen presentation capacity. Thus *P histicola* suppress disease through modulation of Tregs, suppressive macrophages and tolerogenic DCs, which lead to decreased antigen presentation and inflammation.

Milestone #11 Immunomodulatory effect of bacterial treatment was studied by comparison of mRNA expression levels of various cytokines by rtPCR of cytokines in jejenum of treated and untreated mice We have designed the primers for mRNA expression of various cytokine/chemokine expressions in intestines.

Milestone #12 Publication of in vivo data is in progress.

Milestone #13 DR4.TLR4-/- and DR3DQ8.TLR4-/- mice are being characterized.

Milestone #14 and 15 Modulation of cytokines by bacteria in spleen has been studied by using bioplex array system. We have designed of primers for mRNA expression for cytokine/chemokine expression in intestines and analyzed expression levels using RT-PCR.

Reportable Outcome
We have presented our work based on above findings at various international meetings

Abstracts Published at International Meetings:

- David Luckey, Melissa Karau, Robin Patel, Moses Rodriguez, Joseph Murray, Chella David, Veena Taneja and Ashutosh Mangalam (2011). Human commensal bacteria as a novel therapeutic agent for Multiple Sclerosis. Microbial and Mucosal Immunology: the interface in health and disease, San Francisco, CA, USA.

- David Luckey, Marshall Behrens, Melissa Karau, Robin Patel, Ashutosh Mangalam, and Veena Taneja (2011). Microbial Mucosal Modulation of Arthritis. Microbial and Mucosal Immunology: the interface in health and disease, San Francisco, CA, USA.

- Premila Samuel, Arika Wussow, Ashutosh K. Mangalam (2011). The Mechanism of action of Prevotella histocola in the immunomodulation of Experimental autoimmune encephalomyelitis (EAE). 96th Annual Meeting of KAS (Kentucky Academy of Science), Murray State University, Murray, KY, USA.

- Taneja V. Gut and Autoimmunity. " Invited talk" 5th Federation of Immunological Societies Association. New Delhi, India, March 2012.

Publication in scientific Journals

1. Luckey D, BastaKoty D, and **Mangalam A** (2011). Role of HLA class II genes in susceptibility and resistance to multiple sclerosis: studies using HLA transgenic mice. J Autoimmunity 37: 122-128 PMID: 21632210
2. Gomez A, Yoeman C, Luckey D, Marietta EV, Miller ME, Murray JA, White BA and Taneja V. 2012. HLA-DR polymorphism, gut microbiome and sex may predict susceptibility or resistance to arthritis in humanized mice. PloS ONE 7(4):e36095. doi:10.1371 / journal .pone.0036095.

Conclusions. Our data show that P histicola can suppressed ongoing EAE. P histicola treated group showed decreased organ specific pathology in both CIA as well as EAE model. Treatment with P histicola cause decrease infiltration of inflammatory cells in to tissue leading to decreased pathology. We further show that suppressive effect of P histicola is through modulation of regulatory CD4 T cells and tolerogenic DCs as both of these population is increased in treated group. Both Tregs as well as tolerogenic dendritic cells had been shown to suppress inflammatory response. Our experiment on requirement of live vs. dead bacteria for its therapeutic effect is ongoing but our preliminary data suggests that live bacteria is required for therapeutic effect of bacteria as heat killed bacteria did not significantly suppressed disease in one experiment. We are repeating these experiments to confirm these findings. Our ongoing studies on the role of TLR4 will delineate if innate immunity is involved in antigen-specific tolerance in humanized model of arthritis.The results from our ongoing in vivo experiments will help us in understanding if *Prevotella histicola* can be used as a treatment of EAE in HLA-DR3DQ8 transgenic mice and CIA in DR4DQ8 mice.

References

1. Baranzini, S. E., J. Wang, R. A. Gibson, N. Galwey, Y. Naegelin, F. Barkhof, E. W. Radue, R. L. Lindberg, B. M. Uitdehaag, M. R. Johnson, A. Angelakopoulou, L. Hall, J. C. Richardson, R. K. Prinjha, A. Gass, J. J. Geurts, J. Kragt, M. Sombekke, H. Vrenken, P. Qualley, R. R. Lincoln, R. Gomez, S. J. Caillier, M. F. George, H. Mousavi, R. Guerrero, D. T. Okuda, B. A. Cree, A. J. Green, E. Waubant, D. S. Goodin, D. Pelletier, P. M. Matthews, S. L. Hauser, L. Kappos, C. H. Polman, and J. R. Oksenberg. 2009. Genome-wide association analysis of susceptibility and clinical phenotype in multiple sclerosis. *Human molecular genetics* 18:767-778.
2. Mangalam, A., D. Luckey, E. Basal, M. Jackson, M. Smart, M. Rodriguez, and C. David. 2009. HLA-DQ8 (DQB1*0302)-restricted Th17 cells exacerbate experimental autoimmune encephalomyelitis in HLA-DR3-transgenic mice. *J Immunol* 182:5131-5139.
3. Taneja, V., and C. S. David. Role of HLA class II genes in susceptibility/resistance to inflammatory arthritis: studies with humanized mice. *Immunological reviews* 233:62-78.
4. David Luckey, Melissa Karau Robin Patel, Moses Rodriguez, Joseph Murray, Chella David, Veena Taneja and Ashutosh Mangalam. “Human commensal bacteria as a novel therapeutic agent for Multiple Sclerosis” for meeting titled Microbiota and mucosal immunology: the interface in health and disease, April 14-16, 2011, San Francisco, CA, USA
5. David Luckey, Marshall Behrens, Melissa Karau, Robin Patel, Ashutosh Mangalam, and Veena Taneja (2011). Microbial Mucosal Modulation of Arthritis. Microbial and Mucosal Immunology: the interface in health and disease, San Francisco, CA, USA.

Appendices (3 abstracts and one published manuscript)

1. Human commensal bacteria as a novel therapeutic agent for Multiple Sclerosis
Microbiota and mucosal immunology: the interface in health and disease, April 14-16, 2011, San Francisco, CA, USA

David Luckey[1], Melissa Karau[2], Robin Patel[2], Moses Rodriguez[1,3], Joseph Murray[4], Chella David[1], Veena Taneja[1] and **Ashutosh Mangalam[1].**

Department of Immunology, Clinical Microbiology, Neurology, and Gastroenterology, Mayo Clinic, Rochester, MN -55905 USA.

Multiple sclerosis (MS), a chronic inflammatory disease of the CNS, is strongly associated with the MHC class-II genes HLA-DR2, DR3, DR4, DQ8. Here we are proposing a novel approach to cure MS, by administration of a specific strain of human commensal-bacteria. Recent studies have shown that intestinal microflora plays an important role in the health of the host and posses probiotics like qualities. We hypothesize that Gram-negative commensal-bacteria *Prevotella histicola* from human gut have the potential to be used as a therapeutic agent. We have used HLA-DR3DQ8 transgenic mice to test our hypothesis that treatment with commensal-bacteria *P histicola* can modulate experimental autoimmune encephalomyelitis (EAE), an animal model of MS. Previously we showed that PLP_{91-110} can induce EAE in HLA-DR3DQ8 transgenic mice. First using various doses of bacteria, we have identified the optimal dose to be used for treatment of EAE. Our study showed that treatment of mice with 3-4 doses of P. *histicola* in PLP_{91-110}-immunized mice led to suppression of antigen-specific immune response *in-vitro*. Treatment of mice with *P histicola* as probiotics is ongoing. Our data indicates that *P histicola* induced immune responses in the gut cause induction of immune tolerance in periphery leading to suppression of antigen-specific response.

2. Microbial mucosal modulation of arthritis

David Luckey, Marshall Behrens, Melissa Karou, Robin Patel, Joseph Murray, Ashutosh Mangalam and Veena Taneja
Microbiota and mucosal immunology: the interface in health and disease, April 14-16, 2011, San Francisco, CA, USA
Department of Immunology, Gastroenterology and Microbiology, Mayo Clinic Rochester, MN -55901 USA.

Predisposition to rheumatoid arthritis (RA) is associated with the presence of genetic factors, HLA class II molecules, DR4 and DQ8, being the strongest. Recent reports that patients with RA have decreased fecal levels of certain commensal bacteria suggested that intestinal microbes might be critical in regulation of disease. We isolated *Prevotella histicola*, anaerobic commensal bacteria of Human gut, from bowel of a patient and have shown that it possesses anti-inflammatory activity. We propose that gut microbiota can influence peripheral immune response and may modulate arthritis in a murine model. We have established a murine model of rheumatoid arthritis using mice expressing RA-associated HLA genes, DRB1*0401 and DQ8. DR4 and DQ8 mice develop collagen-induced arthritis (CIA) following immunization with type II collagen (CII). We have used HLA-DR4/ DQ8 mice to test our hypothesis that treatment with commensal bacteria like *Prevotella histicola* can modulate CIA. In vitro data showed that treatment of mice with *P. histicola* in CII-immunized mice led to suppression of antigen-specific immune response and reduction in production of inflammatory cytokines suggesting P histicola has anti-inflammatory properties in this model. Treatment of CIA in transgenic mice in therapeutic protocol is

ongoing. Our data suggests that *P histicola* induced immune responses in the gut causes systemic immune suppression and may be able to regulate autoimmunity.

3. The Mechanism of action of *Prevotella histocola* in the immunomodulation of Experimental autoimmune encephalomyelitis (EAE).

96th Annual Meeting of KAS (Kentucky Academy of Science), Murray State University, Murray, KY, USA
PREMILA SAMUEL*[1], Arika Wussow, ASHUTOSH K. MANGALAM[2]. [1]Department of Chemistry, Berea College, Berea, KY 40404. [2]Immunology Department, Mayo Clinic, Rochester, MN 55906.).
Multiple sclerosis (MS) is an inflammatory autoimmune disease affecting the central nervous system. The etiology of MS is extremely complex as both genetic as well as environmental factors may interact in different ways to influence the outcome of disease. In recent year there had been lots of interest in studying the role of gut microbiota as an environmental factors in the immunopathogenesis of inflammatory diseases such as MS. This hypothesis is supported by recent reports that patients with inflammatory diseases have reduced fecal levels of certain commensal bacteria, suggesting possible immunomodulatory role of commensal bacteria in these diseases. Previously, we have shown that human gut specific commensal bacteria, *Prevotella histocola* can suppress Experimental autoimmune encephalomyelitis (EAE), an animal model of MS, in HLA-DR3DQ8 transgenic mice. In this study , we investigated the mechanism of action of immunomodulatory activity of *P histicola* by investigating the ability of *P histicola* to modulate pro and anti-inflammatory cytokine production from LPS stimulated epithelial and dendritic cells. Our preliminary data show that the *in vitro* treatment of Caco-2, a human intestinal epithelial cell line, and THP-1, a human monocytic cell line with *P.histocola* supernatant resulted in the down regulation of pro-inflammatory cytokines TNFα and IL-8 and the up regulation of anti-inflammatory cytokine IL-10. Likewise, the *in vitro* treatment of dendritic cells and macrophages (derived from HLA-DR3.DQ8 double transgenic mice) with *P. histocola* supernatant suppressed the production of TNFα, while inducing the production of IL-10. Thus our preliminary data indicate that *P. histocola* might suppress EAE in HLA class II transgenic mice through modulation of pro and anti-inflammatory cytokines. More detailed studies are underway to understand the detailed mechanism of immunomodulatory action of the commensal bacteria.

Publication in scientific Journals

4. Luckey D, BastaKoty D, and **Mangalam A** (2011). Role of HLA class II genes in susceptibility and resistance to multiple sclerosis: studies using HLA transgenic mice. J Autoimmunity 37: 122-128 PMID: 21632210
5. Gomez A, Yoeman C, Luckey D, Marietta EV, Miller ME, Murray JA, White BA and Taneja V. 2012. HLA-DR polymorphism, gut microbiome and sex may predict susceptibility or resistance to arthritis in humanized mice. PloS ONE 7(4):e36095. doi:10.1371 / journal .pone.0036095.

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Role of HLA class II genes in susceptibility and resistance to multiple sclerosis: Studies using HLA transgenic mice

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ABSTRACT

Multiple sclerosis (MS), an inflammatory and demyelinating autoimmune disease of CNS has both, a genetic and an environmental predisposition. Among all the genetic factors associated with MS susceptibility, HLA class II haplotypes such as DR2/DQ6, DR3/DQ2, and DR4/DQ8 show the strongest association. Although a direct role of HLA-DR alleles in MS have been confirmed, it has been difficult to understand the contribution of HLA-DQ alleles in disease pathogenesis, due to strong linkage disequilibrium. Population studies have indicated that DQ alleles may play a modulatory role in the progression of MS. To better understand the mechanism by which HLA-DR and -DQ genes contribute to susceptibility and resistance to MS, we utilized single and double transgenic mice expressing HLA class II gene(s) lacking endogenous mouse class II genes. HLA class II transgenic mice have helped us in identifying immunodominant epitopes of PLP in context of various HLA-DR and -DQ molecules. We have shown that HLA-DR3 transgenic mice were susceptible to PLP_{91-110} induced experimental autoimmune encephalomyelitis (EAE), while DQ6 (DQB1*0601) and DQ8 (DQB1*0302) transgenic mice were resistant. Surprisingly DQ6/DR3 double transgenic mice were resistant while DQ8/DR3 mice showed higher disease incidence and severity than DR3 mice. The protective effect of DQ6 in DQ6/DR3 mice was mediated by IFNγ, while the disease exacerbating effect of DQ8 molecule was mediated by IL-17. Further, we have observed that myelin-specific antibodies play an important role in PLP_{91-110} induced EAE in HLA-DR3DQ8 transgenic mice. Based on these observations, we hypothesize that epistatic interaction between HLA-DR and -DQ genes play an important role in predisposition to MS and our HLA transgenic mouse model provides a novel tool to study the effect of linkage disequilibrium in MS.

1. Introduction

Multiple sclerosis (MS) is presumed to be an autoimmune disease of the central nervous system (CNS) leading to demyelination, axonal damage, and progressive neurologic disability. Collective evidence suggests that the onset of the disease might result from an aberrant immune response to a number of myelin antigens that is T-cell mediated. The first process of autoimmunity is the peripheral activation of autoreactive CD4+ T cells via the presentation of autoantigens by susceptible MHC class II molecule(s). Therefore it is not surprising that autoimmune diseases such as MS show a strong association with certain HLA class II genes [1–8].

The HLA class II region of the MHC on chromosome 6p21 accounts for the majority of familial clustering in MS and is by far the major susceptibility locus. The class II linkage in MS differs in various populations with the highest association with HLA-DR2 (DRB1*1501)/DQ6 (DQB1*0602) [9–12], Elegant studies by Dyment et al. [4] have shown that the DRB1*17 (DR3) allele is also associated with MS susceptibility. A similar finding on the association of DR3 with MS has been shown in Southern European, Canadian, Mexican and Sardinian MS patients [1,13–15]. Beside DR2/DQ6, DR3/DQ2 and DR4/DQ8 genes are also linked with predisposition to MS [1,12,14,16–18]. Recent studies have shown that disease outcome might be decided by a complex interaction among different class II genes present in a 'haplotype', suggesting that the 'haplotype' might be the basic immunogenetic unit of susceptibility or resistance [3,4,7,8,19].

Although no animal model can mimic all the facets of human MS, the experimental autoimmune encephalomyelitis (EAE) model in rodents has helped immensely in improving our understanding of the immunopathogenesis of MS [20–22]. EAE can be induced in various inbred animal strains by inoculation of whole myelin or defined myelin proteins such as myelin basic protein (MBP), myelin

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oligodendrocytes glycoprotein (MOG), and proteolipid protein (PLP) in complete Freund's adjuvant [20–22]. Elegant studies in murine/rodent EAE have documented that encephalitogenic T cells are CD4+, T helper (Th1)-type cells secreting TNF-α/β and IFNγ [23–25]. However recent studies have indicated that a new T cell phenotype Th17 secreting IL-17, IL-17F, IL-21, IL-22 and IL-23 might also play an important role in the immunopathogenesis of EAE [26]. Thus current hypothesis of EAE indicates that both Th1 and Th17 cytokines play important roles in the immunopathogenesis of EAE.

2. HLA class II transgenic mice expressing HLA-DR or -DQ molecule as an animal model of MS

Despite the fact that MHC genes show the strongest association with MS, the exact role of HLA-DQ and -DR genes in disease pathogenesis is not well understood due to the high polymorphism and heterogeneity of human populations. The strong linkage disequilibrium among HLA-DR, -DQ and other genes within the HLA region makes it difficult to identify the role of individual genes in the immunopathogenesis of MS. In order to understand the role of class II molecules in MS, transgenic mice were generated that express human HLA-DR or -DQ genes lacking endogenous mouse class II genes [27]. A EAE mouse model where the autoreactive T cell repertoire is selected and shaped by human MHC class II molecules has helped us in understanding the immunopathogenesis of inflammatory and demyelinating diseases such as human MS. Using these class II transgenic mice, first we tested whether these human HLA class II molecules are functional by analyzing the T cell proliferative response against various CNS antigens.

2.1. Identification of T cell epitopes

We carried out experiments to determine whether HLA class II molecules in the transgenic mice can efficiently present myelin antigen proteolipid protein (PLP). PLP is the most abundant myelin antigen in CNS and T cells reactive to PLP peptides had been identified in both MS patients and normal controls [28–30]. Using overlapping PLP peptides encompassing the entire sequence of the human PLP molecule (human and mouse PLP are 100% conserved), we identified a number of epitopes restricted to various HLA-DR or -DQ molecules (Table 1). T cell epitopes were spread throughout the entire sequence of PLP molecule [31] and major immunodominant regions were 31–70, 81–120, 140–160, 178–227 and 264–277. Both HLA-DR2 (*1502) and HLA-DR4 (*0401) molecules recognized similar residues on the PLP protein encompassing residues 31–60, 81–120, 178–197, 208–227 and 264–277. The exceptions were $PLP_{51–70}$, recognized only by -DR4 molecule, and residue 198–207 recognized only by -DR2 molecule. HLA-DR3 molecules recognized residues 41–60, 91–110, and 178–227. In summary, all -DR (DR2, DR3 and DR4) and -DQ (DQ6 and DQ8) molecules, recognized regions 41–60, 91–110, 178–197 and 208–227 of PLP. The majority of epitopes identified largely encompassed regions previously reported to be immunogenic in humans [28–30,32]. More importantly not all epitopes were restricted to various class II molecules tested. While, some peptides elicited a response specific to a particular HLA class II allele, others were promiscuous. $PLP_{139–154}$ was immunogenic in DQ6 and DQ8 transgenic mice but not in DR2, DR3 and DR4 transgenic mice. PLP peptide 1–20 was immunogenic only in DQ8 mice. In addition, we observed both DQ and DR restricted response to $PLP_{91–110}$ whereas responses to 264–277 residues of PLP were restricted only to DR molecule. A similar observations have also been reported in a study of MS patients from Japan [32]. Thus HLA class II transgenic mice authenticate restriction of the proteolipid protein (PLP) specific immune response implicated in MS pathogenesis.

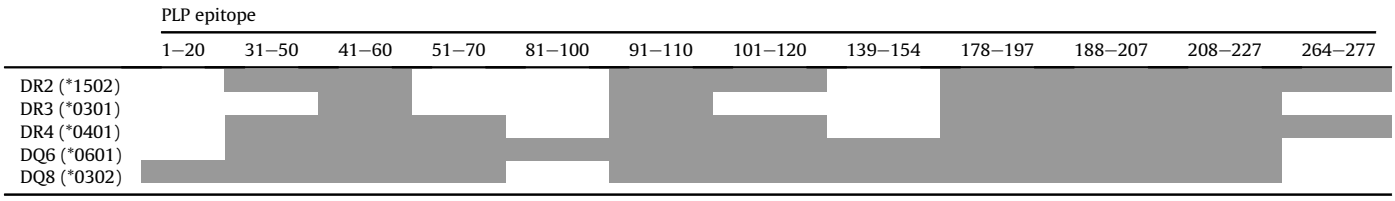
2.2. PLP induced EAE in HLA class II transgenic mice

Our T cell epitope mapping data identified four immunodominant PLP epitopes (PLP 41–60, 91–110, 178–197, and 208–227) in all DR and DQ transgenic mice. Previously, $PLP_{95–116}$ had been shown to be restricted by HLA-DR and -DQ using T cell lines from MS patients while $PLP_{95–116}$ specific T cell clones from HLA-DR2 transgenic mice can induce EAE in Rag2−/− mice. These results support a pathogenic role for $PLP_{95–116}$-specific T cells in HLA-DR2+ MS patients, and shed light on the possible correlation between autoimmune target epitope and disease phenotype in human CNS autoimmune diseases [33].

Based on these studies, we selected $PLP_{91–110}$ peptide for induction of EAE in HLA class II transgenic mice. $PLP_{91–110}$ peptide induced a progressive, chronic EAE in 67% of the HLA-DR3.Aβ° mice [31] and disease was characterized by a typical course of ascending paralysis. The mean onset of the disease was 16 ± 3 days, and maximum disease severity score ranged from 1–3. No clinical sign of disease was seen in transgene negative controls. The majority of affected DR3 mice never went into remission for the length of the test period (10 weeks), and developed a chronic form of EAE. No clinical symptoms were observed in HLA-DR2, -DR4 or HLA-DQ6 or -DQ8 transgenic mice [31]. DR3 transgenic mice with clinical signs of EAE had diffuse meningeal infiltrates in both the spinal cord and the brain. In addition, occasional sections of the spinal cord showed paragonal mononuclear cell infiltrates that were closely associated with the meningeal infiltrates. In the brain, mononuclear cell infiltrates were seen primarily in the meningeal surfaces of the brain stem, cerebellum, and surrounding the ventricles. Small areas of demyelination were observed in spinal cord of DR3 mice with EAE.

Next we examined whether induction of EAE by $PLP_{91–110}$ could lead to intra-molecular or inter-molecular spread of T cell responses to other PLP regions or CNS antigens. Beside $PLP_{91–110}$ induced proliferation responses, T cell proliferation was detected against PLP peptides 141–160, 170–197, 188–207, 208–227, and recombinant MOG, but not MBP protein. Using single amino acid truncation and alanine substitution of encephalitogenic $PLP_{91–110}$, we identified the minimal epitope necessary for binding to the DR3 molecule and to induce EAE [34]. Residues necessary for binding to HLA-DR3 molecule were identified as amino acid 97–108 of PLP. Immunization of DR3 transgenic mice with the minimal epitope

Table 1
Human myelin proteolipid protein specific T cell epitopes recognized by HLA class II molecules.[a]



Each black box represent a positive response to the peptide while white box denote no response to peptide.
[a] Only immunogenic region are shown.

PLP_{97-108} led to induction of EAE and these mice showed classical pathology associated with EAE. The alanine substitutions study showed that residues 99, 102, and 103 are critical for immune recognition of HLA-DR3 molecule [34].

Beside PLP_{91-110} peptide, Ito et al. [35] showed that $PLP_{175-192}$ can induce a strong proliferative response and EAE in HLA-DR4 transgenic mice. Recently using the MBP–PLP fusion protein (MP4) [36], we have identified that $PLP_{178-197}$ peptide can induce EAE in HLA-DR2 (*1502) transgenic mice (Mangalam et al. unpublished observation). Our observation along with previous studies indicate that presence of HLA-DR molecule is required for susceptibility to EAE, as transgenic mice expressing either the human DQ6 or DQ8 genes do not develop disease [27]. Based on these observation, we propose that HLA-DR genes such as HLA-DR2, -DR3 and -DR4 are responsible for predisposition and susceptibility to demyelinating disease, while polymorphism in DQ gene(s) might play a modulating role. This hypothesis is supported by population studies showing that the epistatic interaction between HLA molecules of the disease susceptible haplotypes plays an important role in the final disease outcome in MS. While HLA-DQB1*0601 and -DQB1*0603 protect against MS [3,4,19,37,38], DQB1*0602 and DQB1*0302 alleles can increase disease susceptibility [5,11,37,39,40]. To understand the role of HLA-DQ molecules in the disease process, we generated double transgenic mice expressing HLA-DQ6 or HLA-DQ8 gene on a disease susceptible HLA-DR3 background.

3. HLA-DQ6 (DQB1*0601) suppress EAE in HLA-DR3 transgenic mice by generating anti-inflammatory IFNγ

Human population studies have suggested that HLA-DQ6 (DQB1*0601), found mostly in Asian populations protects from MS [37,41]. To test this protective effect of DQ6 in an experimental model, we generated double transgenic mice expressing both HLA-DR3 and DQ6 on mouse class II negative background. Administration of PLP_{91-110} to DR3.DQ6.Aβ° double transgenic mice with PLP_{91-110} led to disease development only in 40% of double transgenic mice as compared to 70% disease incidence in parental DR3.Aβ° transgenic mice indicating, a protective role of the DQ6 gene [42]. The onset of disease between these two groups was similar. Transgene negative littermates or control Aβ° mice and DQ6 transgenic mice did not develop clinical disease. This clinical disease data suggested that DQ6 plays a protective role by inhibiting development of EAE in disease susceptible DR3 transgenic mice.

Next we analyzed if the protective effect of DQ6 is due to defect in ability of DQ6 molecule to recognize PLP_{91-110} antigen. Interestingly, DR3.DQ6.Aβ° mice showed a very strong, dose dependent T cell response to PLP_{91-110} antigen, which were at least three to four folds higher in magnitude as compared to disease susceptible DR3.Aβ° mice. Similar to double transgenic mice, HLA-DQ6 transgenic mice also showed a very strong T cell proliferative response to PLP_{91-110} as compared to DR3.Aβ° transgenic mice indicating that the DQ6 molecule can present PLP_{91-110} antigen better than DR3.Aβ° transgenic mice. Using the antibody blocking experiment, we confirmed that the strong T cell response observed in DR3.DQ6.Aβ° transgenic mice was restricted to DQ molecule.

As EAE was considered to be Th1 mediated disease, we argued that resistance to EAE in DQ6 mice might be due to expression of Th2 cytokines, which had been shown to be protective in EAE. However, we observed that both the disease resistant DQ6 and protected DR3.DQ6.Aβ° transgenic mice produced high levels of IFNγ, a cytokine normally associated with development of EAE [43,44]. Beside high levels of IFNγ, DR3.DQ6.Aβ° double transgenic mice also produced a moderate level of IL-10 and high levels of IL-2 and IL-27. IL-4 levels were below detection limits in all samples from single and double transgenic mice. In contrast, T cells from disease susceptible DR3.Aβ° transgenic mice produced higher levels of IL-17, IL-22, and IL-23 as compared to DQ6 and DR3.DQ6 mice. We performed an IFNγ-Elispot in the presence or absence of blocking antibodies to confirm that HLA-DQ restricted CD4+ T cells were the source of IFNγ and not CD8 T cells or NK cells.

Our cytokine data indicated that DQ6 restricted IFNγ might be responsible for the protective effect observed in DR3.DQ6.Aβ° transgenic mice. To confirm the role of IFNγ in disease protection, we performed in-vivo studies using neutralizating IFNγ antibody. DR3DQ6 transgenic mice treated with anti-IFNγ but not with isotype control showed increased disease incidence and severity, similar to DR3.Aβ° transgenic mice, confirming a protective role of IFNγ in this model of EAE. Neutralizing antibody treatment in DQ6.Abo mice had no effect. Thus high level of IFNγ plays an anti-inflammatory role and can suppress disease.

IFNγ shows its anti-inflammatory effect through various pathways such as induction of nitric oxide, generation of induced Tregs and apoptosis of antigen specific T cells. PLP_{91-110} specific CD4 T cells from DQ6 mice and DR3DQ6 transgenic mice produced higher level of nitric oxide and had an increased frequency of CD4+FoxP3+ T cells as compared to disease susceptible HLA-DR3 restricted CD4 T cells. We also observed that T cells from DQ6 as well as DR3.DQ6.Aβ° mice undergo increased proliferation and apoptosis as compared to DR3 specific T cells. Thus the protective effect of DQ6 in DR3.DQ6.Aβ° double transgenic mice, was due to high levels of IFNγ produced by DQ6 restricted T cells, which suppressed proliferation of encephalitogenic DR3-restricted T cells by inducing apoptosis. Our study suggests that DQ6 modifies the PLP_{91-110} specific T cell response in DR3 through the anti-inflammatory effects of IFNγ [42].

4. HLA-DQ8 (DQB1*0302) exacerbate disease in HLA-DR3 mice by generating pro-inflammatory IL-17

Presence of the HLA-DQ8/DR4 haplotype has been associated with susceptibility to MS [16–18,45]. To test the role of DQ8 (DQB1*0302) in the immunopathogenesis of MS, we generated HLA class II transgenic mice that express HLA-DQ8 and EAE susceptible DR3 on an MHC II deficient background. Administration of PLP_{91-110} to DR3.DQ8.Aβ° double transgenic mice led to development of disease in 100% of animals as compared to 70% disease incidence in parental DR3.Aβ° transgenic mice, indicating that DQ8 synergizes with DR3 for increased disease penetration. DR3DQ8 double transgenic mice showed an earlier disease onset with increased severity as compared to DR3 transgenic mice (mean clinical score 3.4 ± 0.2 vs. 2.3 ± 0.3, $p < 0.5$). Thus DQ8 plays a modulatory role in DR3.DQ8.Aβ° double transgenic mice by inducing more severe EAE in disease susceptible DR3 transgenic mice [46].

Disease susceptible DR3.Aβ° transgenic mice produced moderate to high levels of IFNγ, TNFα, IL-2, IL-6, and IL-12 cytokines, showing classical Th1 phenotype. Although CD4 T cells from DQ8 mice did not produce IFNγ, they produced significantly higher levels of IL-17 and GM-CSF ($p < 0.01$). Double transgenic DR3.DQ6.Aβ° mice also produced higher levels of IL-17 and GM-CSF as well as IFNγ, besides producing moderate to high levels of TNFα, IL-1, IL-6, and IL-12 cytokines. IL-4 levels were below detection limits in all samples from single and double transgenic mice. DR3.Aβ° transgenic mice also produced moderate amounts of IL-17, IL-21, and IL-23, however, levels were significantly less ($p < 0.01$) as compared to DQ8.Aβ° or DR3.DQ8.Aβ° mice. Both DR3 as well as DR3.DQ8.Aβ° transgenic mice produced comparable levels of IL-27. Thus, disease susceptible DR3.DQ8.Aβ° mice produced higher levels of IL-17, IL-21, IL-23, and IFNγ as compared to DR3 mice.

Using antibody blocking and cytokine Elispot assay, we confirmed that increased levels of IFNγ was produced by DR3 specific T cells, while IL-17 was produced by DQ8 specific T cells. To

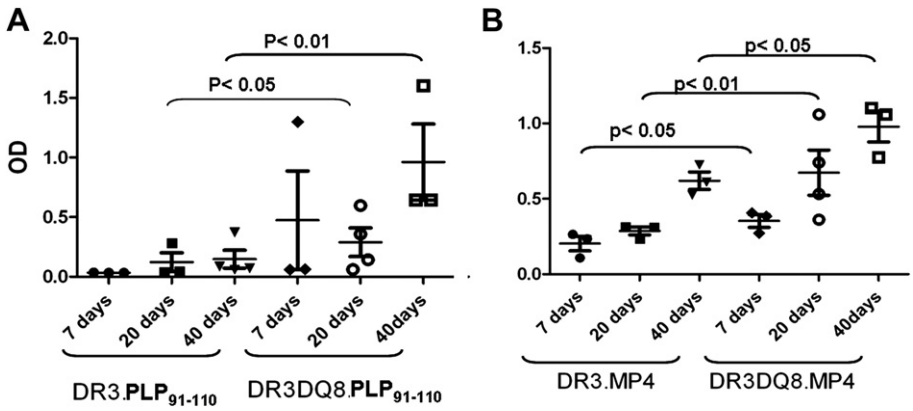


Fig. 1. Antibody response against myelin antigens in HLA class II transgenic mice immunized with PLP_{91-110} peptide. DR3DQ8 mice with EAE showed higher level of antibody against PLP_{91-110} peptide (A) as well as whole PLP–MBP fusion protein (B) compared to DR3 mice with EAE at day 7, 20 and 40 post-immunization. Sera were collected at indicated time points and assayed using PLP_{91-110} or PLP–MBP coated plates using alkaline phosphatase-conjugated (AP) goat anti-mouse IgG (Jackson ImmunoResearch) and p-Nitrophenyl phosphate (PNPP; Southern Biotechnology Associates Inc., Birmingham, AL) as substrate [45].

confirm the respective role of each cytokine in the EAE model, we neutralized either IL-17 or IFNγ in DR3.DQ8.Aβ° mice using specific blocking antibodies and their respective isotype controls. While neutralization of in-vivo levels of IFNγ showed no effect on disease incidence or severity, treatment with neutralizing IL-17 by its specific antibody led to decrease in disease incidence and severity in double transgenic DR3.DQ8.Aβ° mice as compared to mice treated with isotype control antibody [46]. These set of data clearly indicates that the increased disease severity observed in double transgenic DR3.DQ8.Aβ° mice was due to high levels of IL-17 produced by DQ8 specific T cells. Blocking of IFNγ or IL-17 in DQ8.Aβ° mice by neutralizing antibody did not lead to development of disease.

Pathological analysis of brain and spinal cord tissues of mice with EAE showed that DR3.DQ8.Aβ° double transgenic mice developed severe CNS pathology as compared to DR3.Aβ° transgenic mice. DR3.DQ8.Aβ° mice showed more widespread brain pathology with severe inflammation and demyelination in all parts of the brain tissue, including cerebellum, brain stem, cortex, corpus callosum, stratium, and meninges. In contrast, DR3.Aβ° transgenic mice showed inflammation primarily localized to the meninges of the brain. DR3.DQ8.Aβ° transgenic mice with EAE also showed typical parenchymal white matter loss in brain, the classical pathology observed in MS. A similar pattern of pathology was also observed in the spinal cord with increased inflammation and demyelination in DR3.DQ8.Aβ° mice as compared to DR3.Aβ° mice. Thus, DR3.DQ8.Aβ° double transgenic mice develop severe brain and spinal card pathology similar to brain pathology observed in human MS.

5. Myelin-specific antibodies play an important role in PLP_{91-110} induced EAE in HLA-DR3DQ8 transgenic mice

IL-17 can promote autoimmune disease through a mechanism distinct from its pro-inflammatory effects [47]. It has been linked to the induction of autoreactive humoral immune responses because a deficiency in the blockade of IL-17 results in the decline of the autoantibody response [48]. IL-17 can also induce the formation of germinal centers, leading to the activation of B cells and an increased level of antigen presentation and antibody production. It is possible that the IL-17 induced B cells play an important role in the disease exacerbation of DR3.DQ8.Aβ° mice. Further, administration of anti-myelin antibodies has been shown to enhance demyelination in animal models of MS [49–52]. These pathogenic antibodies have been shown to mediate tissue damage by recruitment of classical complement cascade [52,53]. The involvement of

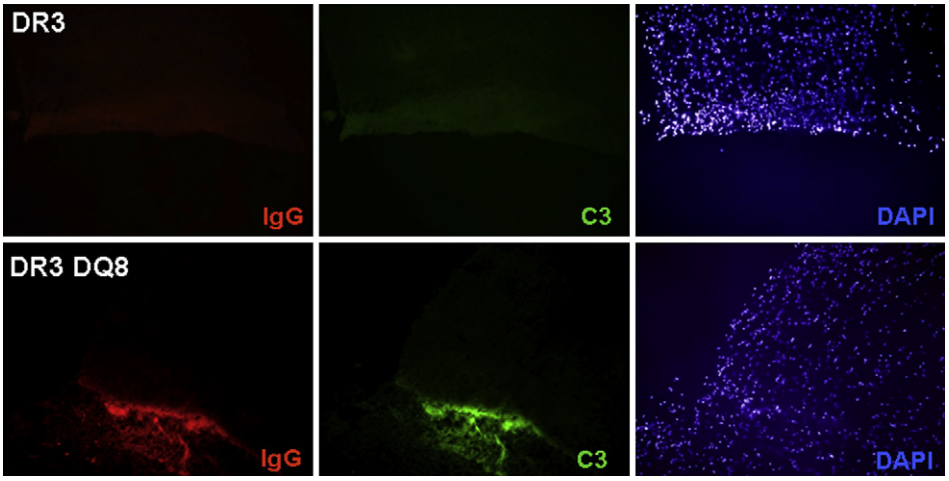


Fig. 2. Immunoflourescence detection of IgG and complement C3 deposition in brain of DR3 and DR3DQ8 mouse with EAE. DR3DQ8 mice with EAE showed higher expression of IgG (red) and C3 deposition (green) in brain section as compared to DR3 mice with EAE. Expression of IgG (red) and C3 (green) were detected by staining brain section with anti-mouse IgG, anti-C3 antibody and fluorescence conjugate secondary antibody. Sections were fixed, counterstained with nuclear DAPI stain and analyzed using fluorescent microscope. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

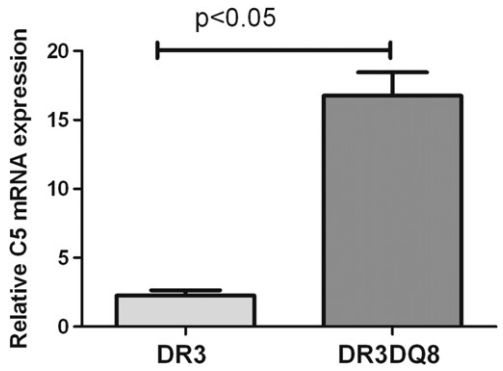


Fig. 3. The relative mRNA levels of C5 in CNS of DR3.Aβ° single and DR3.DQ8.Aβ° double transgenic mice. DR3.DQ8.Aβ° showed higher expression of C5 mRNA as compared to DR3.Aβ° mice. Expressions of C5 mRNA in different transgenic mice were quantified by real-time PCR. Expression of β-actin was used as an internal control. The expression of C5 mRNA in CNS of mice immunized with PLP_{91-110} relative to mice immunized with control PLP peptide was calculated by the ΔΔCt method.

complement-dependent mechanisms in antibody mediated demyelination and pathogenesis in EAE and MS had been noted in several studies [54,55]. Therefore, we investigated the role of anti-myelin antibodies and complement in disease exacerbation and the increased CNS pathology observed in DR3DQ8 mice.

We first analyzed levels of anti-myelin specific antibodies in single and double transgenic mice with EAE. DR3.DQ8.Aβ° double transgenic mice and DR3.Aβ° single transgenic mice were immunized with PLP_{91-110} plus adjuvant as described previously [46] and sera was collected at different time points. Levels of anti-PLP_{91-110} antibodies were detected using ELISA with PLP_{91-110} as the capture antigen. As shown in Fig. 1A, DR3.DQ8.Aβ° double transgenic mice with EAE showed higher levels of anti-PLP_{91-110} antibodies at all time points tested from day 7 to 40 days post-immunization. Levels of anti-PLP_{91-110} IgG increased overtime and maximum levels were observed at day 40 post-immunization. Mice immunized with PLP control peptide or CFA alone showed no reactivity (data not shown). We also tested these sera against the whole PLP–MBP molecules (MP4) using a fusion protein expressing both PLP and MBP protein [36]. Similar to anti-PLP antibodies, we observed that DR3.DQ8.Aβ° double transgenic mice with EAE produced higher levels of anti-MP4 antibodies as compared to DR3.Aβ° single transgenic mice with EAE at all time points (Fig. 1B).

Since, DR3.DQ8.Aβ° double transgenic mice showed severe disease and a higher anti-PLP antibodies level, we hypothesized that increase in clinical and pathological disease might be due to IgG and complement deposition in the CNS leading to tissue destruction. Fresh frozen section were stained with mouse anti-IgG or anti-C3 specific antibody and visualized using fluorescence microscope. As shown in Fig. 2, we observed higher IgG and complement C3 deposition measured in brain sections from DR3.DQ8.Aβ° double transgenic mice with EAE as compared to DR3.Aβ° single transgenic mice with EAE. The deposition was observed in area with inflammatory lesions. We next analyzed expression of C5 mRNA levels in CNS of and observed that the mean C5 mRNA levels in single transgenic DR3 mice were 2 fold higher over control mice(Fig. 3). Whereas in DR3.DQ8.Aβ° mice with EAE, C5 mRNA levels were 15 fold higher than control (7.5 fold increased over single transgenic DR3.Aβ° mice with EAE)(Fig. 3). These data indicate that antibody and compliment play a role in inducing the severe pathology observed in double transgenic mice. These findings are in agreement with earlier reports that anti-myelin antibodies and complement might play an important role in inducing CNS demyelination in MS and EAE [49,52,56–60]. Antibodies against myelin oligodendrocytes glycoprotein (MOG) had been shown to be present within demyelination lesions of cases with acute MS as well as in the marmoset model of EAE [57]. In addition, adoptive transfer of MBP specific CD4 T cells in combination with demyelination monoclonal antibody specific for MOG in Lewis rats has been shown to induce severe disease associated with large plaques of demyelination [58]. Further, pathogenic anti-myelin antibodies had been shown to

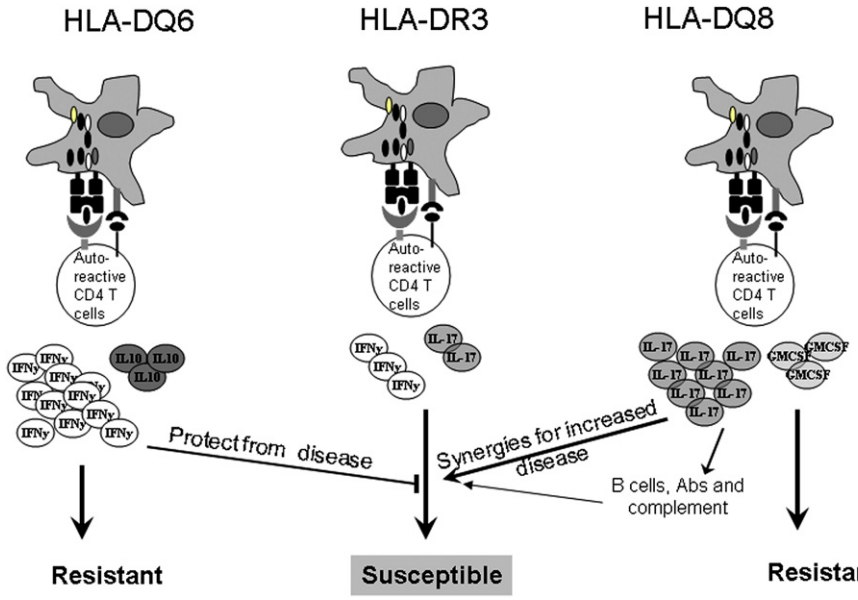


Fig. 4. Schematic diagram of disease susceptibility and resistance in HLA class II transgenic mice and disease modulation by HLA-DQ molecules. HLA-DR3 transgenic mice recognizing PLP_{91-110} peptide produce moderate levels of both Th1 (IFNγ) as well as Th17 (IL-17) cytokines and are susceptible to EAE. Whereas DQ6 mice recognizing the same peptide produce high levels of IFNγ and are resistant to EAE. IFNγ produced by DQ6 restricted T cells is anti-inflammatory as it protects DR3DQ6 from development of EAE. In contrast, DQ8 mice recognizing PLP_{91-110} peptide produce high levels of IL-17 and GM-CSF but are also resistant to development of EAE. Interestingly, presence of DQ8 with DR3 leads to severe disease indicating that DQ8 restricted IL-17 synergizes with DR3 to cause more severe disease. Our recent data show that increased disease and pathology observed in DR3DQ8 double transgenic mice may be due to induction of anti-myelin antibodies by IL-17, which might lead to complement deposition and subsequent neuropathology either through complement mediated cytotoxicity or through antibody dependent cytotoxicity.

induce demyelination through complement activation as mice deficient in C3 develop a milder form of EAE compared to C3 sufficient mice [52,60].

6. Concluding remarks

The main advantage of mouse EAE is that genetically engineered mutants can be generated and bred. Thus, the influence of genetics on susceptibility, disease course, inflammation and demyelination can be studied. An EAE mouse model where the autoreactive T cells repertoire is selected and shaped by human MHC class II molecules will provide new information on immunopathogenesis of inflammatory and demyelinating diseases such as human MS. Thus HLA transgenic mice had been quite useful in understanding the role of HLA class II molecule in immunopathogenesis of EAE/MS. The data from our single and double transgenic mice indicate the final outcome of disease might be dependent on interaction between HLA-DR and -DQ molecules. Utilizing these mice we were able to identifying immunodominant as well as encephalitogenic epitopes of myelin antigen such as PLP, MBP and MOG. Further, data generated from EAE in HLA class II transgenic mice suggest that HLA-DR molecules is required for susceptibility to disease while the HLA-DQ molecule might play a modulatory role through the pro and anti-inflammatory cytokine network(Fig. 4). We have shown that while HLA-DQ6 (DQβ1*0601) can protect DR3 mice from EAE by producing anti-inflammatory IFNγ; HLA-DQ8 (DQβ1*0302) synergize with DR3 to induce a severe disease in DR3DQ8 double transgenic mice by producing pro-inflammatory IL-17 and GM-CSF. Further we have presented data indicating that IL-17 directly or indirectly helps in induction of B cells producing anti-myelin antibodies. We have also shown increased deposition of IgG and complement together with an increased expression of complement in the brain of double transgenic mice with severe EAE. It will be interesting to investigate the mechanism responsible for the production of protective IFNγ from HLA-DQ6 and inflammatory IL-17 from HLA-DQ8 restricted T cells. We join other contributors of this special issue in recognizing the immense contribution of Chella David for his many achievements in the field of autoimmunity including generation of HLA transgenic mice. Advent of these HLA transgenic mice have helped immensely in understanding the immunopathogenesis of various inflammatory, autoimmune, allergic and infectious diseases. Finally, we note that this issue is part of the Journal of Autoimmunity's commitment in the recognition of outstanding scientists in the field of autoimmunity. We are delighted that Chella Davis is being so honored and that he is part of the continuum of distinguished autoimmunologists and dedicated themes in the journal [61–71].

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References

[1] Alvarado-de la Barrera C, Zuniga-Ramos J, Ruiz-Morales JA, Estanol B, Granados J, Llorente L. HLA class II genotypes in Mexican Mestizos with familial and nonfamilial multiple sclerosis. Neurology 2000;55:1897–900.
[2] Barcellos LF, Oksenberg JR, Begovich AB, Martin ER, Schmidt S, Vittinghoff E, et al. HLA-DR2 dose effect on susceptibility to multiple sclerosis and influence on disease course. American Journal of Human Genetics 2003;72:710–6.
[3] DeLuca GC, Ramagopalan SV, Herrera BM, Dyment DA, Lincoln MR, Montpetit A, et al. An extremes of outcome strategy provides evidence that multiple sclerosis severity is determined by alleles at the HLA-DRB1 locus. Proceedings of the National Academy of Sciences of the United States of America 2007;104:20896–901.
[4] Dyment DA, Herrera BM, Cader MZ, Willer CJ, Lincoln MR, Sadovnick AD, et al. Complex interactions among MHC haplotypes in multiple sclerosis: susceptibility and resistance. Human Molecular Genetics 2005;14:2019–26.
[5] Oksenberg JR, Barcellos LF, Cree BA, Baranzini SE, Bugawan TL, Khan O, et al. Mapping multiple sclerosis susceptibility to the HLA-DR locus in African Americans. American Journal of Human Genetics 2004;74:160–7.
[6] Olerup O, Hillert J. HLA class II-associated genetic susceptibility in multiple sclerosis: a critical evaluation. Tissue Antigens 1991;38:1–15.
[7] Ramagopalan SV, Deluca GC, Morrison KM, Herrera BM, Dyment DA, Lincoln MR, et al. Analysis of 45 candidate genes for disease modifying activity in multiple sclerosis. Journal of Neurology 2008;255:1215–9.
[8] Ramagopalan SV, Morris AP, Dyment DA, Herrera BM, DeLuca GC, Lincoln MR, et al. The inheritance of resistance alleles in multiple sclerosis. PLoS Genetics 2007;3:1607–13.
[9] McDonald WI. Multiple sclerosis: epidemiology and HLA associations. Annals of the New York Academy of Sciences 1984;436:109–17.
[10] Oksenberg JR, Begovich AB, Erlich HA, Steinman L. Genetic factors in multiple sclerosis. Jama 1993;270:2362–9.
[11] Barcellos LF, Sawcer S, Ramsay PP, Baranzini SE, Thomson G, Briggs F, et al. Heterogeneity at the HLA-DRB1 locus and risk for multiple sclerosis. Human Molecular Genetics 2006;15:2813–24.
[12] Dyment DA, Sadovnick AD, Ebers GC. Genetics of multiple sclerosis. Human Molecular Genetics 1997;6:1693–8.
[13] Cullen CG, Middleton D, Savage DA, Hawkins S. HLA-DR and DQ DNA genotyping in multiple sclerosis patients in Northern Ireland. Human Immunology 1991;30:1–6.
[14] Marrosu MG, Murru MR, Costa G, Cucca F, Sotgiu S, Rosati G, et al. Multiple sclerosis in Sardinia is associated and in linkage disequilibrium with HLA-DR3 and -DR4 alleles. American Journal of Human Genetics 1997;61:454–7.
[15] Masterman T, Ligers A, Olsson T, Andersson M, Olerup O, Hillert J. HLA-DR15 is associated with lower age at onset in multiple sclerosis. Annals of Neurology 2000;48:211–9.
[16] Weinshenker BG, Santrach P, Bissonet AS, McDonnell SK, Schaid D, Moore SB, et al. Major histocompatibility complex class II alleles and the course and outcome of MS: a population-based study. Neurology 1998;51:742–7.
[17] Marrosu MG, Muntoni F, Murru MR, Spinicci G, Pischedda MP, Goddi F, et al. Sardinian multiple sclerosis is associated with HLA-DR4: a serologic and molecular analysis. Neurology 1988;38:1749–53.
[18] Zivadinov R, Uxa L, Bratina A, Bosco A, Srinivasaraghavan B, Minagar A, et al. HLA-DRB1*1501, -DQB1*0301, -DQB1*0302, -DQB1*0602, and -DQB1*0603 alleles are associated with more severe disease outcome on MRI in patients with multiple sclerosis. International Review of Neurobiology 2007;79:521–35.
[19] Chao MJ, Barnardo MC, Lincoln MR, Ramagopalan SV, Herrera BM, Dyment DA, et al. HLA class I alleles tag HLA-DRB1*1501 haplotypes for differential risk in multiple sclerosis susceptibility. Proceedings of the National Academy of Sciences of the United States of America 2008;105:13069–74.
[20] Gold R, Hartung HP, Toyka KV. Animal models for autoimmune demyelinating disorders of the nervous system. Molecular Medicine Today 2000;6:88–91.
[21] Martin R, McFarland HF, McFarlin DE. Immunological aspects of demyelinating diseases. Annual Review of Immunology 1992;10:153–87.
[22] Swanborg RH. Experimental autoimmune encephalomyelitis in the rat: lessons in T-cell immunology and autoreactivity. Immunological Reviews 2001;184:129–35.
[23] Ando DG, Clayton J, Kono D, Urban JL, Sercarz EE. Encephalitogenic T cells in the B10.PL model of experimental allergic encephalomyelitis (EAE) are of the Th-1 lymphokine subtype. Cell Immunology 1989;124:132–43.
[24] Kuchroo VK, Anderson AC, Waldner H, Munder M, Bettelli E, Nicholson LB. T cell response in experimental autoimmune encephalomyelitis (EAE): role of self and cross-reactive antigens in shaping, tuning, and regulating the autopathogenic T cell repertoire. Annual Review of Immunology 2002;20:101–23.
[25] Zamvil SS, Steinman L. The T lymphocyte in experimental allergic encephalomyelitis. Annual Review of Immunology 1990;8:579–621.
[26] Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 cells. Annual Review of Immunology 2009;27:485–517.
[27] Mangalam AK, Rajagopalan G, Taneja V, David CS. HLA class II transgenic mice mimic human inflammatory diseases. Advances in Immunology 2008;97:65–147.
[28] Greer JM, Csurhes PA, Cameron KD, McCombe PA, Good MF, Pender MP. Increased immunoreactivity to two overlapping peptides of myelin proteolipid protein in multiple sclerosis. Brain 1997;120(Pt 8):1447–60.
[29] Martino G, Hartung HP. Immunopathogenesis of multiple sclerosis: the role of T cells. Current Opinion in Neurology 1999;12:309–21.
[30] Schmidt S. Candidate autoantigens in multiple sclerosis. Multiple Sclerosis (Houndmills, Basingstoke, England) 1999;5:147–60.
[31] Mangalam AK, Khare M, Krco C, Rodriguez M, David C. Identification of T cell epitopes on human proteolipid protein and induction of experimental autoimmune encephalomyelitis in HLA class II-transgenic mice. European Journal of Immunology 2004;34:280–90.
[32] Minohara M, Ochi H, Matsushita S, Irie A, Nishimura Y, Kira J. Differences between T-cell reactivities to major myelin protein-derived peptides in opticospinal and conventional forms of multiple sclerosis and healthy controls. Tissue Antigens 2001;57:447–56.
[33] Kawamura K, Yamamura T, Yokoyama K, Chui DH, Fukui Y, Sasazuki T, et al. HLA-DR2-restricted responses to proteolipid protein 95-116 peptide cause autoimmune encephalitis in transgenic mice. The Journal of Clinical Investigation 2000;105:977–84.

[34] Mangalam AK, Khare M, Krco CJ, Rodriguez M, David CS. Delineation of the minimal encephalitogenic epitope of proteolipid protein peptide(91-110) and critical residues required for induction of EAE in HLA-DR3 transgenic mice. Journal of Neuroimmunology 2005;161:40–8.
[35] Ito K, Bian HJ, Molina M, Han J, Magram J, Saar E, et al. HLA-DR4-IE chimeric class II transgenic, murine class II-deficient mice are susceptible to experimental allergic encephalomyelitis. The Journal of Experimental Medicine 1996;183:2635–44.
[36] Elliott EA, McFarland HI, Nye SH, Cofiell R, Wilson TM, Wilkins JA, et al. Treatment of experimental encephalomyelitis with a novel chimeric fusion protein of myelin basic protein and proteolipid protein. The Journal of Clinical Investigation 1996;98:1602–12.
[37] Amirzargar A, Mytilineos J, Yousefipour A, Farjadian S, Scherer S, Opelz G, et al. HLA class II (DRB1, DQA1 and DQB1) associated genetic susceptibility in Iranian multiple sclerosis (MS) patients. European Journal of Immunogenetics 1998;25:297–301.
[38] Herrera BM, Ebers GC. Progress in deciphering the genetics of multiple sclerosis. Current Opinion in Neurology 2003;16:253–8.
[39] Das P, Drescher KM, Geluk A, Bradley DS, Rodriguez M, David CS. Complementation between specific HLA-DR and HLA-DQ genes in transgenic mice determines susceptibility to experimental autoimmune encephalomyelitis. Human Immunology 2000;61:279–89.
[40] Serjeantson SW, Gao X, Hawkins BR, Higgins DA, Yu YL. Novel HLA-DR2-related haplotypes in Hong Kong Chinese implicate the DQB1*0602 allele in susceptibility to multiple sclerosis. European Journal of Immunogenetics 1992;19:11–9.
[41] Marrosu MG, Muntoni F, Murru MR, Costa G, Pischedda MP, Pirastu M, et al. HLA-DQB1 genotype in Sardinian multiple sclerosis: evidence for a key role of DQB1 *0201 and *0302 alleles. Neurology 1992;42:883–6.
[42] Mangalam A, Luckey D, Basal E, Behrens M, Rodriguez M, David C. HLA-DQ6 (DQB1*0601)-restricted T cells protect against experimental autoimmune encephalomyelitis in HLA-DR3.DQ6 double-transgenic mice by generating anti-inflammatory IFN-gamma. Journal of Immunology 2008;180:7747–56.
[43] Steinman L. A rush to judgment on Th17. The Journal of Experimental Medicine 2008;205:1517–22.
[44] Stromnes IM, Cerretti LM, Liggitt D, Harris RA, Goverman JM. Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. Nature Medicine 2008;14:337–42.
[45] Khare M, Mangalam A, Rodriguez M, David CS. HLA DR and DQ interaction in myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis in HLA class II transgenic mice. Journal of Neuroimmunology 2005;169:1–12.
[46] Mangalam A, Luckey D, Basal E, Jackson M, Smart M, Rodriguez M, et al. HLA-DQ8 (DQB1*0302)-restricted Th17 cells exacerbate experimental autoimmune encephalomyelitis in HLA-DR3-transgenic mice. Journal of Immunology 2009;182:5131–9.
[47] Hsu HC, Yang P, Wang J, Wu Q, Myers R, Chen J, et al. Interleukin 17-producing T helper cells and interleukin 17 orchestrate autoreactive germinal center development in autoimmune BXD2 mice. Nature Immunology 2008;9:166–75.
[48] Komiyama Y, Nakae S, Matsuki T, Nambu A, Ishigame H, Kakuta S, et al. IL-17 plays an important role in the development of experimental autoimmune encephalomyelitis. Journal of Immunology 2006;177:566–73.
[49] Piddlesden SJ, Lassmann H, Zimprich F, Morgan BP, Linington C. The demyelinating potential of antibodies to myelin oligodendrocyte glycoprotein is related to their ability to fix complement. The American Journal of Pathology 1993;143:555–64.
[50] Raine CS, Cannella B, Hauser SL, Genain CP. Demyelination in primate autoimmune encephalomyelitis and acute multiple sclerosis lesions: a case for antigen-specific antibody mediation. Annals of Neurology 1999;46:144–60.
[51] Schluesener HJ, Sobel RA, Linington C, Weiner HL. A monoclonal antibody against a myelin oligodendrocyte glycoprotein induces relapses and demyelination in central nervous system autoimmune disease. Journal of Immunology 1987;139:4016–21.
[52] Urich E, Gutcher I, Prinz M, Becher B. Autoantibody-mediated demyelination depends on complement activation but not activatory Fc-receptors. Proceedings of the National Academy of Sciences of the United States of America 2006;103:18697–702.
[53] Pollinger B, Krishnamoorthy G, Berer K, Lassmann H, Bosl MR, Dunn R, et al. Spontaneous relapsing-remitting EAE in the SJL/J mouse: MOG-reactive transgenic T cells recruit endogenous MOG-specific B cells. The Journal of Experimental Medicine 2009;206:1303–16.
[54] Du C, Sriram S. Increased severity of experimental allergic encephalomyelitis in lyn-/- mice in the absence of elevated proinflammatory cytokine response in the central nervous system. Journal of Immunology 2002;168:3105–12.
[55] Rus H, Cudrici C, Niculescu F, Shin ML. Complement activation in autoimmune demyelination: dual role in neuroinflammation and neuroprotection. Journal of Neuroimmunology 2006;180:9–16.
[56] Cross AH, Stark JL. Humoral immunity in multiple sclerosis and its animal model, experimental autoimmune encephalomyelitis. Immunologic Research 2005;32:85–97.
[57] Genain CP, Cannella B, Hauser SL, Raine CS. Identification of autoantibodies associated with myelin damage in multiple sclerosis. Nature Medicine 1999;5:170–5.
[58] Linington C, Engelhardt B, Kapocs G, Lassman H. Induction of persistently demyelinated lesions in the rat following the repeated adoptive transfer of encephalitogenic T cells and demyelinating antibody. Journal of Neuroimmunology 1992;40:219–24.
[59] Martin Mdel P, Monson NL. Potential role of humoral immunity in the pathogenesis of multiple sclerosis (MS) and experimental autoimmune encephalomyelitis (EAE). Frontiers in Bioscience 2007;12:2735–49.
[60] Szalai AJ, Hu X, Adams JE, Barnum SR. Complement in experimental autoimmune encephalomyelitis revisited: C3 is required for development of maximal disease. Molecular Immunology 2007;44:3132–6.
[61] Youinou P. Haralampos M. Moutsopoulos: a lifetime in autoimmunity. Journal of Autoimmunity 2010;35:171–5.
[62] Miyagawa F, Gutermuth J, Zhang H, Katz SI. The use of mouse models to better understand mechanisms of autoimmunity and tolerance. Journal of Autoimmunity 2010;35:192–8.
[63] Scheinecker C, Bonelli M, Smolen JS. Pathogenetic aspects of systemic lupus erythematosus with an emphasis on regulatory T cells. Journal of Autoimmunity 2010;35:269–75.
[64] Youinou P, Pers J-O. The international symposium on Sjogren's syndrome in Brest: the "top of the tops" at the "tip of the tips". Autoimmunity Reviews 2010;9:589–90.
[65] Lu Q, Renaudineau Y, Cha S, Ilei G, Brooks WH, Selmi C, et al. Epigenetics in autoimmune disorders: highlights of the 10th Sjogren's syndrome symposium. Autoimmunity Reviews 2010;9:627–30.
[66] Youinou P, Pers J-O, Gershwin ME, Shoenfeld Y. Geo-epidemiology and autoimmunity. Journal of Autoimmunity 2010;34:J163–7.
[67] Chang C, Gershwin ME. Drugs and autoimmunity - A contemporary review and mechanistic approach. Journal of Autoimmunity 2010;34:J266–75.
[68] Borchers AT, Naguwa SM, Keen CL, Gershwin ME. The implications of autoimmunity and pregnancy. Journal of Autoimmunity 2010;34:J287–99.
[69] Schilder AM. Wegener's granulomatosis vasculitis and granuloma. Autoimmunity Reviews 2010;9:483–7.
[70] Ansari AA, Gershwin ME. Navigating the passage between Charybdis and Scylla: recognizing the achievements of Noel Rose. Journal of Autoimmunity 2009;33:165–9.
[71] Mackay IR. Clustering and commonalities among autoimmune diseases. Journal of Autoimmunity 2009;33:170–7.

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Loss of Sex and Age Driven Differences in the Gut Microbiome Characterize Arthritis-Susceptible *0401 Mice but Not Arthritis-Resistant *0402 Mice

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Abstract

Background: HLA-DRB1*0401 is associated with susceptibility, while HLA-DRB1*0402 is associated with resistance to developing rheumatoid arthritis (RA) and collagen-induced arthritis in humans and transgenic mice respectively. The influence of gut-joint axis has been suggested in RA, though not yet proven.

Methodology/Principal Findings: We have used HLA transgenic mice carrying arthritis susceptible and -resistant HLA-DR genes to explore if genetic factors and their interaction with gut flora gut can be used to predict susceptibility to develop arthritis. Pyrosequencing of the 16S rRNA gene from the fecal microbiomes of DRB1*0401 and DRB1*0402 transgenic mice revealed that the guts of *0401 mice is dominated by a Clostridium-like bacterium, whereas the guts of *0402 mice are enriched for members of the *Porphyromonadaceae* family and *Bifidobacteria*. DRB1*0402 mice harbor a dynamic sex and age-influenced gut microbiome while DRB1*0401 mice did not show age and sex differences in gut microbiome even though they had altered gut permeability. Cytokine transcripts, measured by rtPCR, in jejuna showed differential TH17 regulatory network gene transcripts in *0401 and *0402 mice.

Conclusions/Significance: We have demonstrated for the first time that HLA genes in association with the gut microbiome may determine the immune environment and that the gut microbiome might be a potential biomarker as well as contributor for susceptibility to arthritis. Identification of pathogenic commensal bacteria would provide new understanding of disease pathogenesis, thereby leading to novel approaches for therapy.

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Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that is characterized by synovial inflammation and erosion of bone and cartilage leading to the destruction of joints. Although the etiology of RA is unknown, both genetic and environmental factors contribute to the susceptibility to developing arthritis [1]. Among the known genetic factors, strong associations are observed between RA and the presence of certain HLA-DR alleles that share the 3rd hypervariable region with DRB1*0401 gene, known as the 'shared epitope' hypothesis. In contrast, DRB1*0402 confers resistance to the development of arthritis. Some evidence points to an infectious etiology for RA, such the presence of certain oral and gut commensal bacterial antigens in synovial fluids of patients [1,2,3]. Migration of gut commensals or their products to peripheral organs may be facilitated by loss of intestinal integrity, resulting in mucosal or systemic immune stimulation. Recent studies have shown that specific intestinal commensals or their specific molecular patterns may modulate the integrity of the intestinal mucosal barrier by inducing the expression of pro or anti-inflammatory cytokines [4,5]. Thus, alterations of a normal gut microbiome can affect mucosal immunity and have an extended effect on non-intestinal diseases like diabetes and RA [6]. For instance, previous analysis of the fecal microbiome of patients with RA revealed significantly fewer *Bifidobacterium* and bacteria of the *Bacteroides-Porphyromonas-Prevotella* group, *B. fragilis* subgroup, and the *Eubacterium rectale–Clostridium coccoides* group than the fecal microbiota of patients with non-inflammatory fibromyalgia [7]. Because these bacterial species are known to belong to common taxa in the human fecal microbiome [8,9,10], their low levels in RA patients might suggest an altered gut microbiome. Further, specific gut commensals such as *Bifidobacterium infantis*, can induce an anti-inflammatory response in the intestinal mucosal and peripheral immune systems by suppressing T-cell proliferation and production of IL-10 and Th2 cytokines, and by inhibiting nuclear factor kappa B (NF-κB) activation [11,12,13]. Although dendritic

cells (DCs), directly in contact with intestinal lumen contents, can instruct naive CD4+ T-cells to differentiate into Th1, Th17, Th2 or T-regulatory cells, a unique gastro-intestinal environment may favor the proliferation of the latter, a process possibly dependent on the presence of specific gut commensal bacteria thus setting up the basis for immunotolerance [14,15,16].

We have generated two lines of HLA transgenic mice carrying the RA-susceptible DRB1*0401 and RA-resistant DRB1*0402 genes that lacked all four classical murine chains, Aα, Aβ, Eα, Eβ. Because human class II molecules shape the T-cell repertoire in these humanized mice, they show the same HLA restrictions in an immune response as humans [17,18,19]. Upon immunization with type II collagen (CII), *0401 mice develop collagen-induced arthritis (CIA), while *0402 mice do not. The most remarkable features of CIA in *0401 mice that is not observed in any other model is a sex-bias in the onset of arthritis with a ratio of 3 Females: 1 Male, production of rheumatoid factor (RF) and anti-cyclic citrullinated peptide antibodies (ACPAs), diagnostic markers for RA patients [18,20]. Host MHC genes affect the microbial composition of the gut [21,22]. However, the interactions between host genetic factors like MHC and their gut microbiota, and their impact on the development of RA are difficult to study in humans due to several factors that include: high HLA polymorphism, diet and the fact that the disease is well established at the time of diagnosis. Few studies describing microbiome of RA patients have not done tag sequencing and also not analyzed the data according to sex and age. Thus, HLA transgenic mice described here provide a useful tool to understand the role of gut microbiota in the pathogenesis of RA. Herein, we show that mice with the RA-susceptible DRB1*0401 gene harbor altered patterns of gut microbiome characterized by an abundance and/or lack of specific commensals as compared to mice with the RA-resistant DRB1*0402 gene whose gut microbiomes are shaped by age and sex. A differential expression of Th17 regulating gene transcripts, a compromised gut permeability in *0401 mice and observed dysbiosis in *0401 mice may in combination or independently contribute to susceptibility to arthritis.

Results

Arthritis-susceptible DRB1*0401 and –resistant DRB1*0402 mice differ in their gut microbiomes

The gut microbiome plays a crucial role in the homeostasis of the immune system and is also linked to gut permeability. We tested if an arthritis-susceptible genotype may be associated with the presence or absence of specific gut bacteria by sequencing the microbiome community structure in fecal samples of 87 mice (n = 41 for *0401 and n = 45 for *0402 mice) using Roche 454 GS-FLX Titanium Pyrosequencing technology. This included both male and female mice of various ages for both strains. After processing, 568,571 high quality sequences were used for further analysis (sequence lengths ranged from 417 to 534 bp with a 506 median sequence length). A total of 5,267 operational taxonomic units (OTUs) clustered at 97% sequence similarity were used for microbiome analysis (1,953 to 60,915 reads per sample).

Non-metric multidimensional scaling (NMDS) and analysis of similarities (ANOSIM) suggest that *0401(n = 41) and *0402 (n = 45) mice display only minor differences in their fecal microbiome profiles (ANOSIM R-statistic = 0.14, P = 0.001) (Figure 1 a). There were no significant differences in bacterial richness between the 2 strains (P>0.1). However, the fecal bacterial species in *0401 mice were slightly more evenly distributed than those in *0402 mice (Shannon evenness index, P = 0.04). Both NMDS and ANOSIM analysis of males and females from each strain showed that sex was a confounding factor and that males were masking the differences between the fecal bacterial profiles of resistant and susceptible mice (Figure 1 b); the ANOSIM R value between *0401 (n = 22) and *0402 (n = 21) males was only 0.145 (P<0.001). In contrast, differences in fecal microbiome structure were more evident between *0401 (n = 19) and *0402 (n = 24) females (ANOSIM R statistic = 0.436, P<0.001), (Figure 1 c and d). DRB1*0402 mice showed dynamically different fecal microbiomes based on sex (males and females, n = 21 and 24 respectively and age (<4 and >4 months old, n = 27 and n = 18 respectively) factors (ANOSIM R-statistic = 0.302 and 0.423 for sex and age differences respectively, P<0.001) (Figure 2a and 2b). Unlike arthritis-resistant *0402 mice however, the structure of the fecal microbiomes of *0401 mice lost these sex (males and females, n = 22 and n = 19 respectively) and age (<4 and >4 months old, n = 30 and n = 11 respectively) - driven differences (ANOSIM R values of 0.052 and 0.043 for gender and age differences respectively, P>0.1) (Figures 3 c and d).

Specific gut commensals contribute to strain, sex and age differences in mice fecal microbiomes

A percentages-species contribution analysis (SIMPER) and Taxonomic search using RDP and NCBI databases allowed us to identify and characterize the relative abundance distributions of the five Operational Taxonomic Units (OTUs) that contributed more than 2.5% to the observed differences of the fecal microbiomes between resistant and susceptible transgenic mice (Table 1, Figure 3a). The phyla distributions of OTUs followed similar patterns when taking into account all of the 5,267 OTUs detected (Figure 3 b); at this taxonomic level, *0401 mice presented a more even Bacteroidetes/Firmicutes ratio (~1:1) than *0402 mice (~2:1).

An OTU related to *Allobaculum* sp. (84% identity to *A. stercoricanis*) or an unclassified member of the Clostridiales (87% identity) was more abundant in disease-susceptible (*0401) mice compared to *0402 mice (P<0.00001). On the other hand, OTUs related to *Bifidobacterium*, *Barnesiella* and *Parabacteroides* spp., were more abundant in disease-resistant mice (P = 0.0029) (Figure 3 a). Data on the relative abundance of the OTU's driving microbiome differences between the two strains were used to construct a simple correspondence analysis plot (CA) showing the level of correlation between each OTU and members of either strain (Figure 4 c). Dimension 1 (Axis CA1) of the CA plot explained 42.94% of the total variation in the data and distinguished between susceptible mice, more correlated to the abundance of *Allobaculum* sp. and resistant mice, associated to greater proportions of the *Bifidobacteria* and the *Parabacteroides-Barnesiella* group.

Sex based differences in the fecal microbiomes of *0402 mice were driven mainly by *Bifidobacterium pseudolongum* subsp. *Globosum* and *Parabacteroides distasonis*, each being more prevalent in females (P = 0.018. and 0.00017 for *Bifidobacterium* and *Parabacteroides* respectively) (Figure 4a) while, *Barnesiella viscericola* was more abundant in males (P = 0.00018. n = 21 and 24 for resistant male and female mice respectively). Dimension 1 of a CA plot describing the level of association between either sex and specific OTUs explained 57.29% of the total variation in the data and showed high correlation between the relative abundance of *Barnesiella viscericola* and *0402 males, and of *Bifidobacterium pseudolongum and Parabacteroides distasonis* and *0402 females (Figure 4 b). Even though fecal microbiome of *0401 mice were dysbiotic and less dynamic (Figure 4 d), susceptible males did show significantly higher abundances of *B. pseudolongum* than susceptible females (P = 0.02, n = 22 and 19 for *0401 males and females

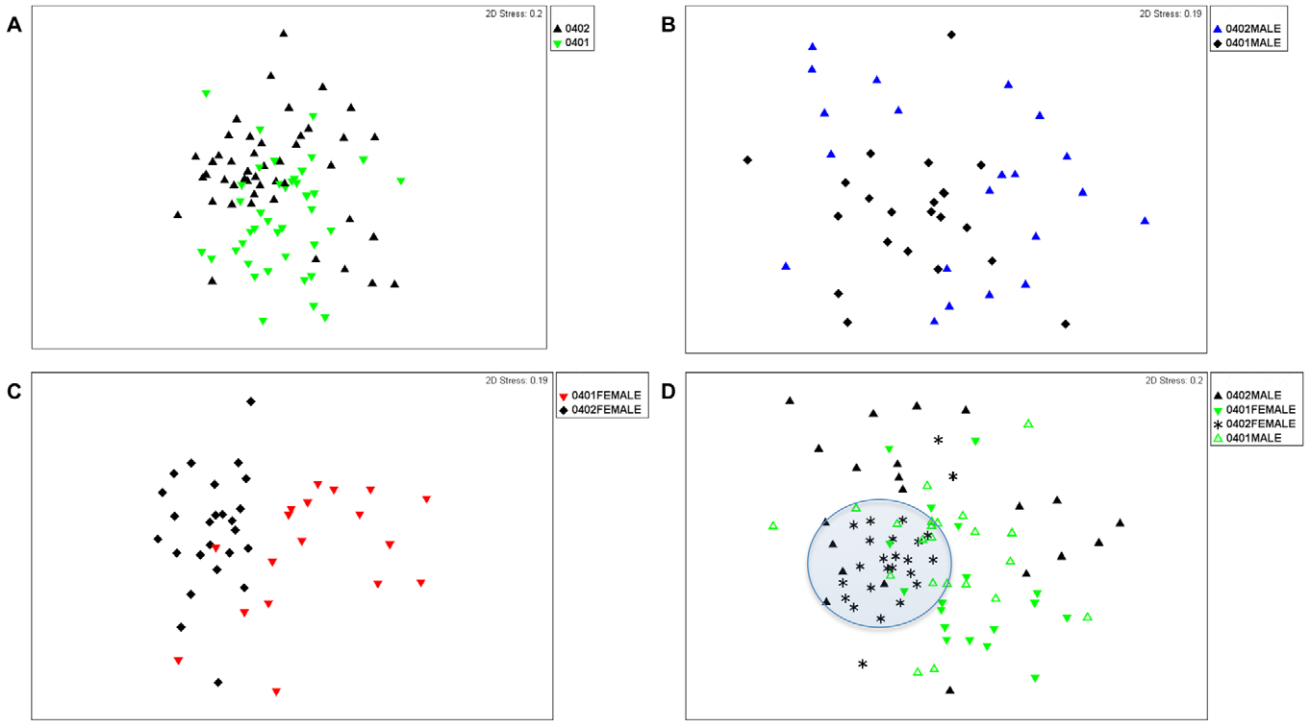

Figure 1. Multidimensional Scalinge analysis of the fecal microbiomes of arthritis-susceptible *0401 and –resistant *0402 mice. 16S-rDNA bacterial community structure differences can be visualized with each symbol representing data from a single mouse fecal sample. (**a**) 16S-rDNA bacterial community structures between *0401 (n = 41) and *0402 (n = 45) mice do not differ significantly (ANOSIM R = 0.14). (**b**) *0401 (n = 22) and *0402 (n = 21) males do not show significant differences in fecal microbiome structure (ANOSIM R = 0.14), while (**c**) fecal microbiomes of *0401 (n = 19) and *0402 (n = 24) females differ significantly (ANOSIM R = 0.436). (**d**) Shaded area shows that the fecal microbiomes of *0402 females are compact and may be driving differences between both genotypes.
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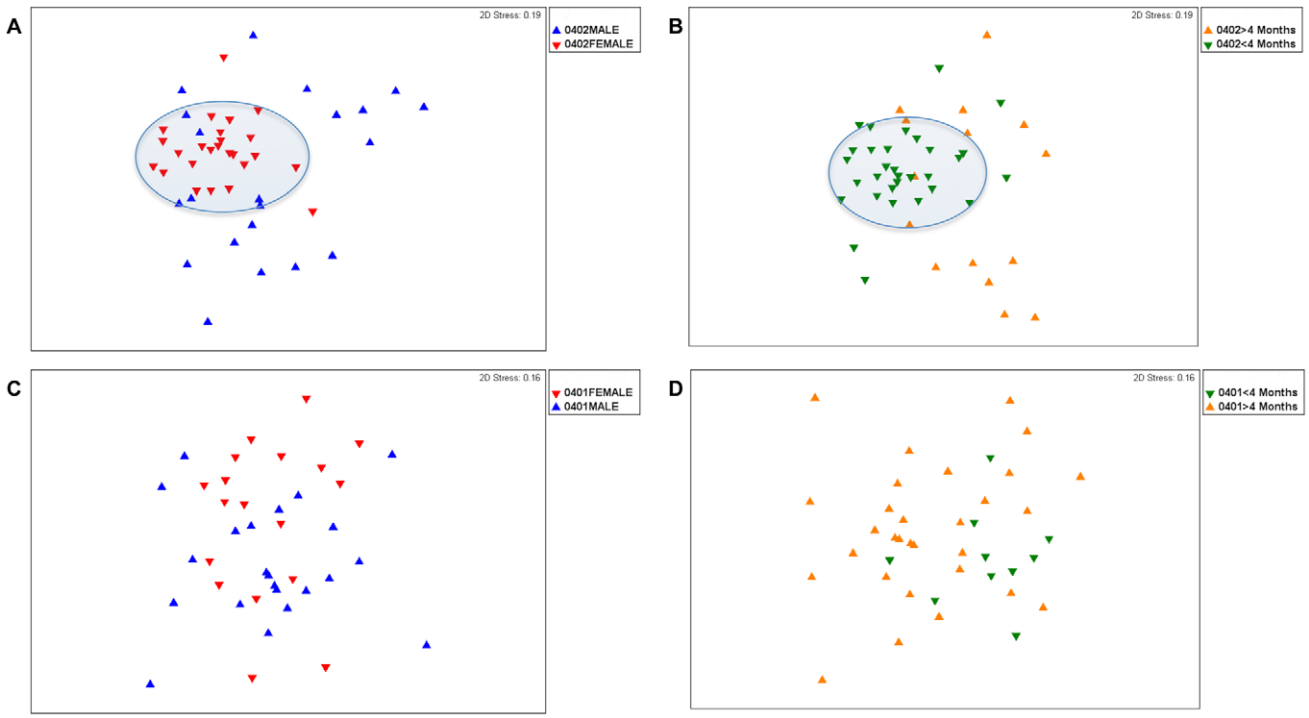

Figure 2. Sex and Age based Multidimensional Scalinge analysis of fecal microbiomes. (**a**) *0402 mice show significantly different fecal microbiome structure according to sex (ANOSIM R = 0.302) and (**b**) age (ANOSIM R = 0.423). (**c**) *0401 mice lost sex and (**d**) age-based differences in fecal microbiome (ANOSIM R = 0.052 and R = 0.043 respectively). Shaded areas show compact microbiome structures.
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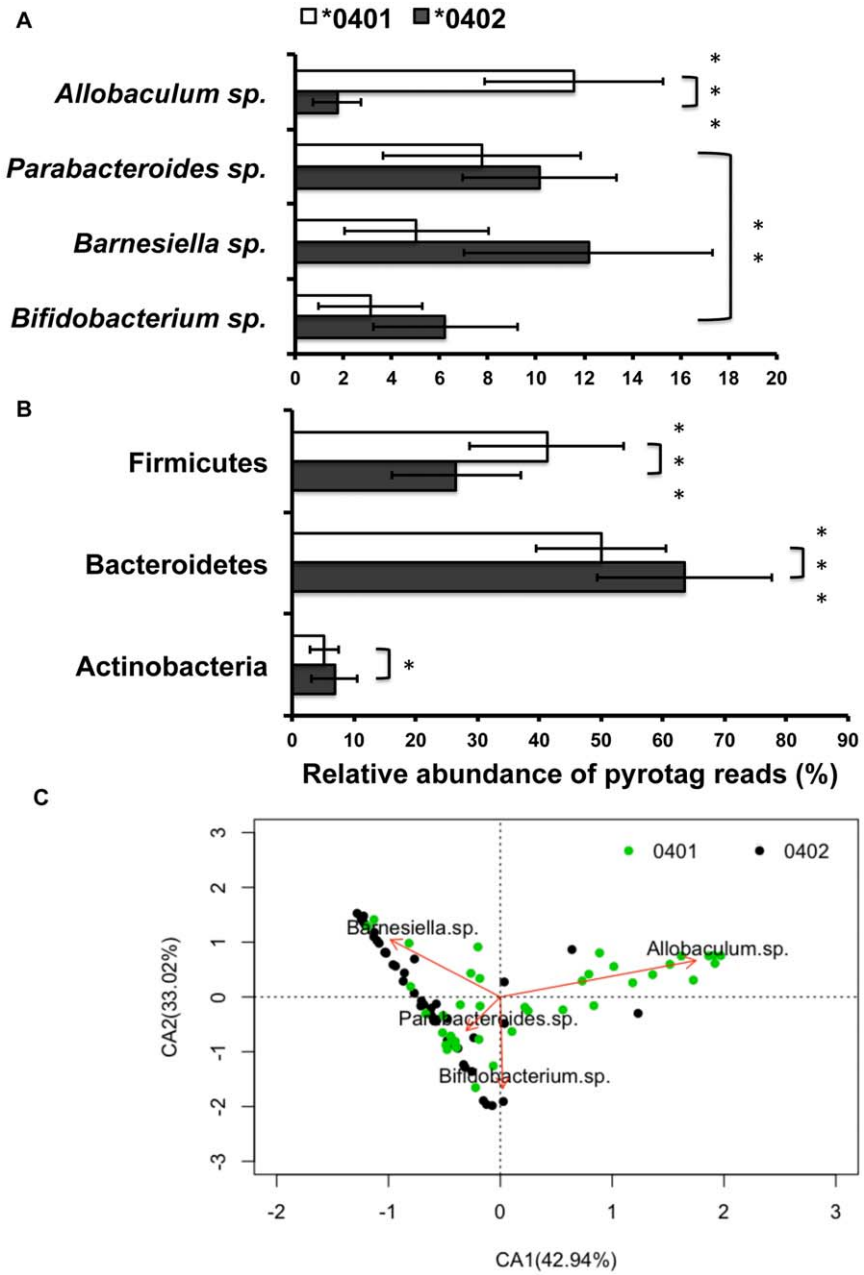

Figure 3. Relative abundance of OTUs in the fecal microbiomes of *0401 and *0402 mice. (**a**) *Allobaculum sp*. is is the most abundant OTU in the *0401 mice, while *Barnesiella* sp. occurs with highest frequency in *0402 mice ($P<0.05$). (**b**) Similar taxa distributions are observed at phyla level with Bacteroidetes: Firmicutes ratios more even in *0401 (n = 41) compared to *0402 (n = 45) mice. Data are presented as mean ± S.E., *$P<0.05$, **$P<0.01$, ***$P<0.001$. (**c**) Correspondence analysis plot shows the degree of correlation between specific OTUs and mice genotype. The red vectors point to the center of gravity of the samples where each OTU mostly occurs. The distance between the tip of the vector and the samples (dots) give an indication of the probability of OTU content in each sample. Green and black dots represent *0401 and *0402 fecal samples respectively. Percentages in parentheses in the CA plots describe the amount of variation explained by each axis.
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respectively) (Figure 4 c). Age dependent fecal microbiota differences in resistant mice were driven mainly by *B. viscericola* that more abundant in older mice (<4 compared to >4 months, n = 18 and 27 respectively, P = 0.0001). However, the relative abundances of *Bifidobacterium* and *Parabacteroides* were not significantly different between older and younger *0402 mice (P = 0.766 and P = 0.0567 for *Bifidobacterium* and *Parabacteroides* respectively. There were no significant age-driven differences in the relative abundance of specific OTUs between susceptible mice.

Arthritis susceptible DRB1*0401 mice show altered mucosal immune function and increased gut permeability compared to resistant DRB1*0402 mice

We tested the hypothesis that dysbiosis in gut flora of *0401 mice may be associated with an altered intestinal permeability as well as a distinct expression of pro and anti-inflammatory cytokines in the gut as compared to arthritis-resistant mice, and that this dysbiosis may play a role in the pathogenesis of arthritis. A comparison of gut permeability between arthritic and naïve

Table 1. Identity of the main OTUS's driving strain, age and gender differences among mice fecal microbiomes.

Phylogenetic Affiliation	Closest match in the RDP environmental sequence database and refseq_rna	% ID	Closest match in the nr/nt NCBI database	% ID	Closest uncultured match in the nr/nt NCBI database	% ID	No. of times detected in the data set
Actinobacteria	*Bifidobacterium pseudolongum* subsp. *globosum* strain JCM 5820	99	*Bifidobacterium pseudolongum* subsp. *globosum* strain 02-2	100	Uncultured Bifidobacterium sp. clone PP187-b15	100	36,261
	NR_043441.1		AY166515.1		GU902754.1		
Bacteroidetes	*Barnesiella intestinihominis* YIT 11860 strain YIT	84	Gram-negative bacterium cL10-2b-4	88	Uncultured bacterium clone RMAM0391	99	30,891
	NR_041668.1		AY239469.1		HQ319321.1		
Bacteroidetes	*Barnesiella viscericola* DSM 18177 strain JCM 13660	82	Gram-negative bacterium cL10-2b-4	87	Uncultured bacterium gene	95	35,585
	NR_041508.1		AY239469.1		AB626927.1		
Bacteroidetes	*Parabacteroides distasonis* strain JCM 5825	83	Gram-negative bacterium cTPY-13	89	Uncultured bacterium clone 16saw38-2d06.q1k	99	35,234
	NR_041342.1		AY239461.1		EF604593.1		
Firmicutes	*Allobaculum stercoricanis* DSM 13633	84	Bacterium HBND	87	Uncultured bacterium clone R-9085	89	34,042
	NR_042110.1		AY498748.1		FJ881077.1		

Phylotype identity was assigned using the RDP (Ribosomal Database Project) classifier at 80% Bayesian bootstrap cutoff from comparisons to the environmental survey sequence database. RNA entries from NCBI's Reference Sequence project (refseq_rna) and no longer "non-redundant" nucleotide collection of the NCBI were also used to assign phylotype.

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*0401 mice showed a significant increase in gut permeability in arthritic (n = 5) mice compared to naïve (n = 5) mice ($P<0.0001$, Figure 5a). To determine if the host genotype and gut flora may determine gut permeability, naïve male and female *0401 and *0402 mice were kept on a similar diet, cage bedding and room. Our data showed that there is a basal level of intestinal permeability which is significantly higher in *0401 mice as compared to *0402 mice and that it is age and sex-dependent in susceptible mice (Figure 5b). There was no difference in gut permeability between sexes at a young age (<4 months); however, as the *0401 mice aged, females (>4 months age) showed an increase in gut permeability as compared to the younger group, $P<0.04$ and older *0402 females ($P<0.03$). Resistant mice did not show any significant changes in gut permeability with age or sex (n = 5–8 in each group).

To determine if gut microbial composition is also associated with a different gut immune profile, we tested the jejuna of naïve mice for expression of cytokine and chemokine transcripts involved in the Th17 regulatory network by rtPCR (Figure 6 a–e, Figure S1, File S1). Susceptible *0401 females showed a distinct cytokine and chemokine profile as compared to males that was characterized with a significant increase in IL-23α and IFNγ along with a decrease in the regulatory cytokines IL-4, IL-22 and CCL20. Similarly, *0401 females showed more than 3 fold increased gene transcripts for Th17 cytokines IL-17, IL-23, IL-6 and Th1 cytokines IFNγ, Stat 4 and TBX21 while *0402 females had several fold increase in genes regulating Th2 cytokines and regulatory networks like ICOS, GATA3 and IL-4. *0401 male mice did not show an increase in transcripts for TH17 encoding genes compared to *0402 mice.

Next we determined if the relative abundance of the OTUs showing sex, age and strain differences in the transgenic mice were associated with specific cytokine/chemokine transcripts in jejuna (n = 12, 3 mice from each group, *0401 and *0402 males and females). Spearman correlation tests showed that *Bifidobacterium* species were negatively correlated to IL-17a ($P = 0.06$) and TBx21 ($P = 0.004$) transcript levels, while *Parabacteroides* species were negatively correlated to TBx21 ($P = 0.012$) (Table S1). The relative abundance of *Allobaculum* species were negatively correlated to CCL22 ($P = 0.017$) and IL-21 ($P = 0.06$). Since cytokines transcripts were studied in only six mice/strain, these results need to be interpreted with caution.

Discussion

Interaction between genetic and environmental factors is required for predisposition to develop RA. The presence of bacterial DNA of gut-residing commensals in synovial fluid [3] has led to the hypothesis that certain mucosal bacteria may have a role in the susceptibility to develop arthritis. By taking advantage of transgenic mice that express the RA-susceptible *0401 transgene or the RA-resistant *0402 transgene, we have shown that genetic factors, along with sex background and disruption of gut microbiome may influence susceptibility and/or resistance toward developing arthritis in humanized mice. Our data on gut microbiome in genetically resistant mice is consistent with previous reports of age and sex based differences in the fecal microbiomes of healthy individuals [23,24]. Resistant transgenic female mice, whose microbiomes were more similar, drove differences in microbiome structure between the two strains. This suggests that host genotype, rather than sex background, is a major regulator of gut microbial composition, an observation consistent with a recent report in inbred lines of mice [25]. However, as different human and murine models have shown, it is under debate whether the

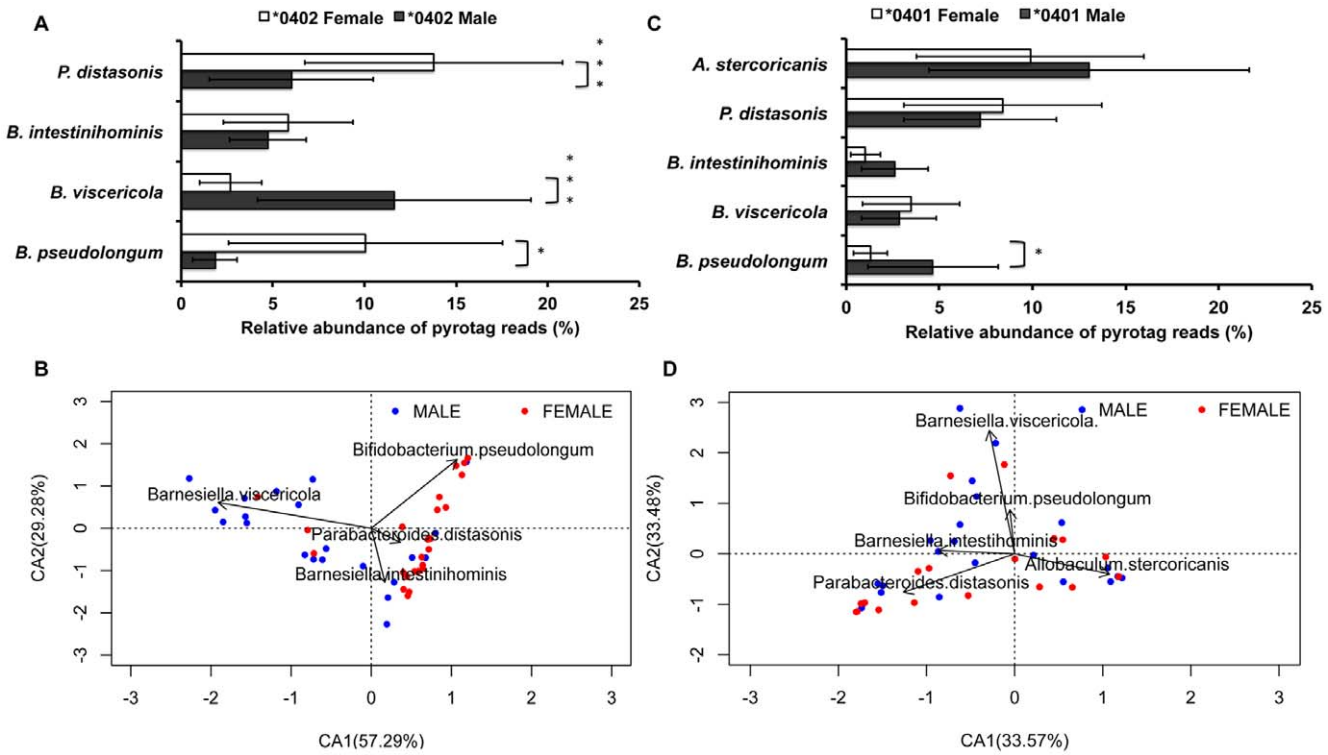

Figure 4. Sex based relative abundance of OTUs in the fecal microbiomes of *0401 and *0402 mice. (**a**) *0402 females (n = 24) show significantly higher relative abundances of *Bifidobacterium-Parabacteroides* OTUs compared to males (n = 21), whose microbiomes present significantly higher levels of *Barnesiella viscericola*. (**b**) Correspondence analysis plot displays sex-based correlation between OTUs in *0402 mice. (**c**) Significantly higher relative abundances of *Bifidobacterium sp*. were observed in *0401 males (n = 22) compared to females (n = 19), (**d**) despite loss of dynamic sex based differences in the fecal microbiomes of *0401 mice. Percentages in parentheses in the CA plots describe the amount of variation explained by each axis. Data are presented as mean ± S.E. *P<0.05, **P<0.01, ***P<0.001.
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maintenance of adaptive immune mechanisms are mainly applied top-bottom (host-controlled) or bottom-up (driven by the gut microbiome), with gut bacterial communities acting as puppets or masters of the immune system [4,26,27,28]. In this respect, the findings presented herein imply that the selection of a different T cell repertoire by two distinct HLA transgenes [29] modulates gut bacterial communities. This is consistent with studies that show increases in the incidence of autoimmune disorders driven by genotype, in such studies, interactions with specific commensals, harmless under immunocompetent conditions, could trigger disease [30,31]. Conversely, studies with germ-free and specific pathogen-free mice have shown that disruptions to gut bacteria can promote increased levels of pro-inflammatory cytokine and interlukin-17 producing Th-17 cells, even in tissues distant to the gut [27,31,32], suggesting that global adaptive immune responses are also controlled by gut bacteria. Our data showed a bias towards TH1/TH17 cytokine expression with significant decrease in cytokine gene transcripts required for negative regulation of Th17 profile, like IL-4, IL-21 and IL-22, in *0401 females as compared to *0401 males and *0402 females. Interestingly CCL20 and CCL22 which are required for the generation of regulatory CD4 T cells and DCs [33,34,35], are reduced several fold in *0401 females as compared to *0401 males and *0402 females. A recent study showed that decreased levels of CCL20 during aging, are

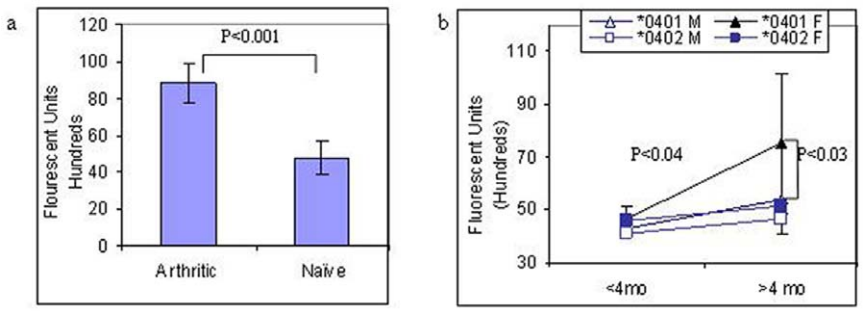

Figure 5. Gut permeability. (**a**) Transgenic arthritic mice showed significantly higher gut permeability compared to naïve mice (n = 5 each group). (**b**) Intestinal permeability in naïve *0401 and *0402 transgenic male and female mice at >4 and <4 months of age. *0401F<4 mo vs >4 mo, P<0.04; *0401F vs *0402F >4 mo, P<0.03 (n = 5–8 in groups).
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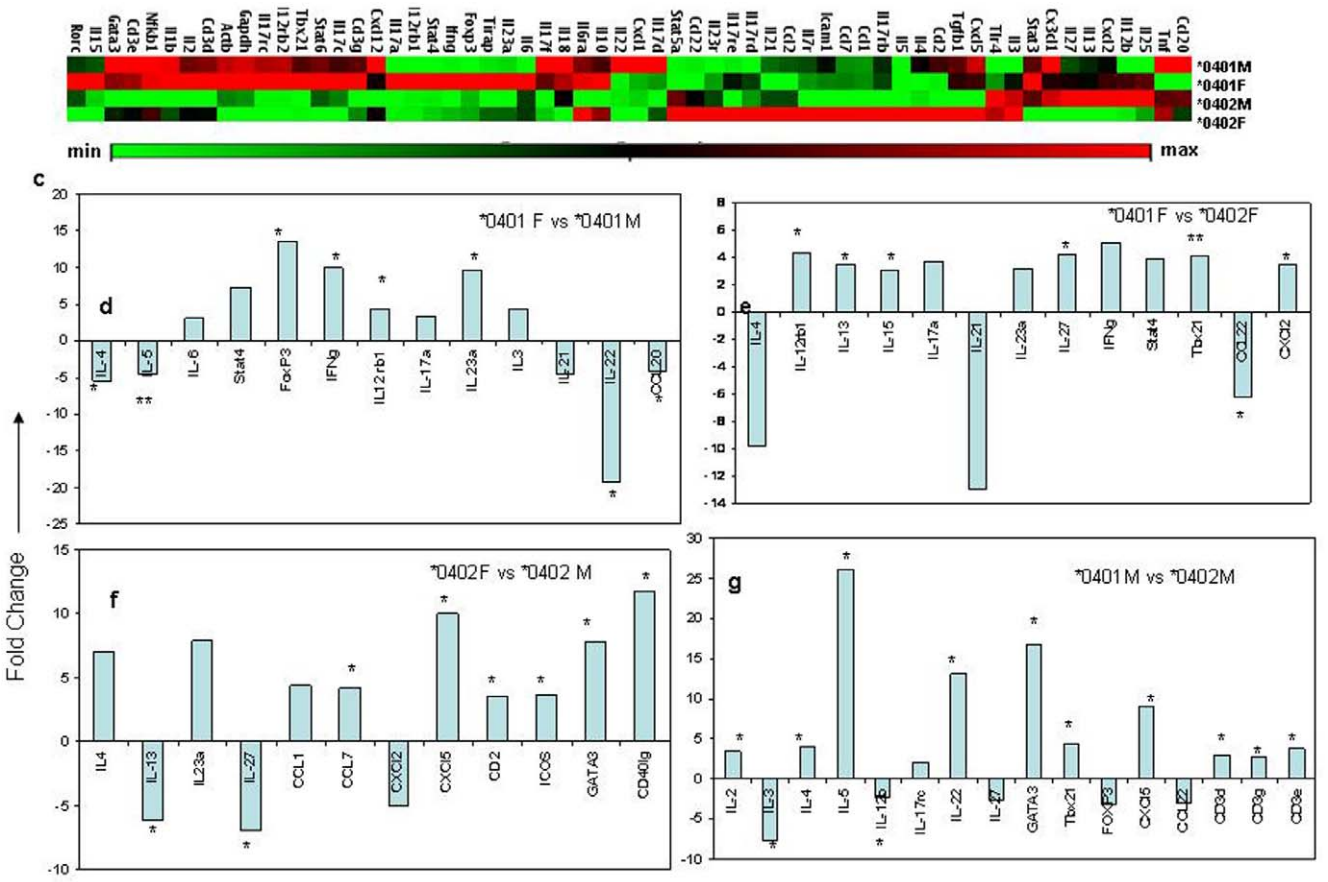

Figure 6. A)) Heat map showing expression levels of cytokines and chemokine transcripts in jejunum of *0401 and *0402 male and female mice (n = 3 in each group). (b) Comparison of fold change in gene transcript levels between *0401 females and males, (c) *0402 females and males, (**d**) females of each genotype and (e) males of each genotype. Results are given as fold-changes of mean copy-numbers relative to the mean copy-numbers of the comparative group. *P<0.05 and **P<0.01 and more. Data points with 3 or more fold differences and significance of more than P<0.05 are shown.
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associated with isolated lymphoid dysfunction and mucosal immunosenescence [35]. These studies along with the present results that show a decreased CCL20 and loss of age differences in gut microbiome of *0401 females may confer mucosal dysfunction and immunosenescence. Arthritis-susceptible males however, showed a significant increase in Th1/Th2 but not in Th17 cytokines compared to resistant males, along with significant increases in genes for γδ T cells, suggesting a role for these cells in Th1/Th2 profile. Differences in chemokine gene transcripts observed between *0401 and *0402 mice and their correlation with microbiome profiles further supported our contention of dysbiosis leading to altered mucosal immune function in susceptible mice. However, these events could be contributing to pathogenesis independtly or in combination.

The significantly higher evenness index and even Bacteroidetes/Firmicutes ratios in *0401 as compared to *0402 mice, and their specific associations with either genotype, may be a reflection of an apparent dysbiosis phenomenon similar to that observed in other disease conditions [36]. Our observations suggest that *0402 mice maintain a homeostatic gut bacterial environment characterized by the overrepresentation and/or absence of specific microbiome structure. Specifically, members of Bacteroidetes and Actinobacteria occur twice as often as Firmicutes in *0402 mice as opposed to the stable ratios observed in *0401 mice. This observation implies that host genotype and environmental stimuli can cause expansion and/or contraction of certain members of a core or signature gut microbiome to modulate immunity. In the present study, microbiome differences between arthritis-susceptible and resistant mice are higher relative abundance of *Bifidobacterium pseudolongum* and members of the *Porphyromonadaceae* family in the latter that are positively correlated with regulatory cytokines. These observations support the importance of these taxa in the maintenance of a homeostatic gut microbiome. *Bifidobacteria* sp. have been recognized for their probiotic and immuno-modulating properties including down-regulating the expression of inflammatory pathways and enhancing gut barrier function [4,36,37,38]. *Parabacteroides distasonis*, a commensal detected in higher proportions in arthritis-resistant mice, has been reported to reduce intestinal inflammation in murine models upon oral administration of its antigens [39] and is also involved in "educating" the immune system towards the tolerance of commensal antigens by enhancing Treg cell recognition mechanisms [40]. Thus in *0401 mice, particularly in females, reduced relative abundance of *Bifidobacterium sp.* and commensals from the *Porphyromonadaceae* family may lead to dysbiosis, enhanced pro-inflammatory responses, and a subsequent skewed immune response. Interestingly, the proportions of *Bifidobacterium* are inversely proportional or negatively correlated to the presence of *Allobaculum* sp. (Clostridiales order). Segmented filamentous bacteria (SFB), also from the Clostridiales, have been linked to immunosuppression

and an increase of pro-inflammatory responses in arthritis driven by Th-17 cell proliferation [41,42]. Although SFB were not detected in this study, the gut microbiomes of *0401 mice were characterized by a 7 fold increase in the relative abundance of *Allobaculum* sp. compared to *0402 mice. Based on 16S rRNA gene phylogenetic analysis, *A. stercoricanis* forms a branch closer to the XVI Clostridial cluster constituted by *Clostriudium innocum*, *Streptococcus pleomorphus* and several *Eubacterium* spp. [43]. In fact *C. innocum* was the closest hit in RDP database (80% ID), which could imply high phylogenetic concordance of our sequence to members of this Clostridiales group. Consistent with our results, *Clostridium* spp. have been reported to be enriched in immune-compromised subjects [44] and reductions in *C. innocuum* levels are reported upon oral administration of *Bifidobacterium* spp. [45]. A broader search in the non-redundant nucleotide NCBI database, also related this Clostridium-like sequence to a bacterium isolated from mice deficient in secretory antibodies (87% identity), in which there was increased recognition of gastrointestinal tract flora antigens by systemic antibodies and increased bacterial translocation [46]. Thus, these findings may indicate an association between inflammation and the high abundance of this OTU in *0401 mice.

Our data in humanized mice is also supported by a study in early RA patients, in which lower levels of Bifidobacteria and bacteria of the Bacteroides-Porphyromonas-Prevotella group were observed in RA patients compared to non-arthritic patients [7]. Members of the *Porphyromonadaceae* family are common dwellers of intestinal, oral and urogenital human and murine flora and have been identified as opportunistic commensals potentially pathogenic after immune disruption [47]. Herein, the relative abundance of members of the *Porphyromonadaceae* family (*Barnesiella and Parabacteroides* spp.) were significantly reduced in susceptible mice whenever the Clostridia-like bacterium became abundant. This observation implies that the Clostridia-like bacterium may induce disruption of normal commensals that are non-pathogenic under immunocompetent conditions. An increase in the gut permeability has been suggested to play a role in pathogenesis of arthritis [48]. Thus, the differences in microbiome and gut permeability seen in the *0401 and *0402 raises the possibility that, under certain conditions, disease causing bacteria like Clostridia, and other pathobionts or symbionts could produce translocation and a systemic immune response resulting in arthritis in those with a genetic susceptibility.

An interesting observation is the dynamic different microbiome structures based on age and/or sex background, driven by specific bacterial groups in *0402 mice. In contrast, this kind of microbial axis dynamism is completely lost in susceptible mice. This suggests the execution of specific, age/sex-based, immune regulation mechanisms in resistant mice. In this case, only females may have benefited from relative high abundance of *Bifidobacteria and Parabacteroides*. However, both *0402 males and females, regardless of age, seem to benefit from an absence of the Clostridia-like bacterium. This observation, along with the fact that in *0401 mice males may be taking advantage of significantly higher abundance of Bifidobacteria compared to females, raises the hypothesis that it is the loss of bacterial dynamism that is associated with disease susceptibility, particularly in females. A similar situation can be envisaged in human models in which beneficial microbiome may act as a modulator of proper immune response in the absence of host-genetic immune-regulators, and presence of pro-inflammatory bacteria potentially triggering disease. Although, specific molecular mechanisms remain largely unexplored, together, these results suggest that susceptibility to RA could be triggered by gut dysbiosis in genetically susceptible individuals. In turn, the onset of resistance may be characterized by a more dynamic microbiome, whose members expand and/or contract to provide sex and/or age based competent immune function, especially, in individuals that are not prone to develop RA. The hypotheses proposed herein (Figure 7), could be tested in future studies through experimentation with germ-free and SPF mice using various ways to manipulate gut microbiome and measure its impact in triggering disease. These approaches may include administration of probiotics which have been shown to alter intestinal microbiota and immune response and suppress CIA [49,50]. Additionally, this model points a way forward to further probe gut microbial communities in various disease conditions, which would allow us to identify novel biomarkers and develop preclinical models to manipulate them for therapeutics.

Materials and Methods

Transgenic mice

The generation of DRB1*0401and DRB1*0402 transgenic (Tg) mice has been described previously [20,29]. Aβ°.DRB1*0401 and Aβ°.DRB1*0402 mice were mated with MHCII$^{\Delta/\Delta}$ (AE°) mice [51] to generate AE°.DRB1*0401 and AE°.DRB1*0402 mice. Mice of both sexes (8–12 weeks of age) used in this study were bred and maintained in the pathogen-free Immunogenetics Mouse Colony at the Mayo Clinic, Rochester, MN in accordance with the Institutional Animal care and use Committee (IACUC). For convenience, DRB1*0402 mice will be referred to as *0402, and DRB1*0401 mice as *0401.

The expression of DR on PBLs of transgenic mice was analyzed by flow cytometry using mAbs L227 (anti-DR) conjugated antibodies to characterize transgene positive mice.

16 s rDNA analysis of Mice Fecal microbiome

Microbial DNA was extracted using the MoBio UltraClean Soil Kit (Mo Bio Laboratories Inc., Carlsbad, CA, USA) with a bead-beating step from fecal material of a single mouse. The V1–V3 region of the 16S ribosomal RNA gene was amplified by Polymerase chain reaction (22 cycles of 94°C (30 s), 48°C (30 s), 72°C (2 min)) using primers 27f (CGTATCGCCTCCCT-CGCGCCATCAG-AGAGTTTGATYMTGGCTCAG; corresponding to nucleotides 8–27 of the Escherichia coli 16 s rRNA gene) and 534r (CTATGCGCCTTGCCAGCCCGCTCAG-[MID tag 1–15]-ATTACCGCGGCTGCTGGCA; corresponding to nucleotides 514–534 of the E. coli 16 s rRNA gene). The amplicons were subjected to pyrosequencing using 454 FLX-Titanium technologies at the UIUC KECK. The resulting sequences were processed using a combination of tools from Mothur [52] and custom Perl scripts. Preliminary quality control steps included the removal of sequences shorter than 400 nt with homopolymers longer than 6 nucleotides and all reads containing ambiguous base calls or incorrect primer sequences. Sequences were aligned against the silva database and then trimmed so subsequent analyses were constrained to the same portion of the 16S rDNA. Potentially chimeric sequences were detected using chimera slayer (http://www.mothur.org/wiki/chimera.slayer/) and removed. The remaining reads were pre-clustered to remove sequences that are likely to have derived from sequencing errors (http://www.mothur.org/wiki/Pre.cluster) and then clustered using Mothur's average algorithm. Taxonomic classification of each OTU (clustered at 97% sequence similarity) was obtained by Blastn alignments to NCBI RNA reference sequence and non-redundant nucleotide databases and with the Ribosomal Database project (RDP) multiclassifier at 80% Bayesian bootstrap cutoff from comparisons to the environmental survey sequence database. All new data were deposited in the sequence read archive (http://

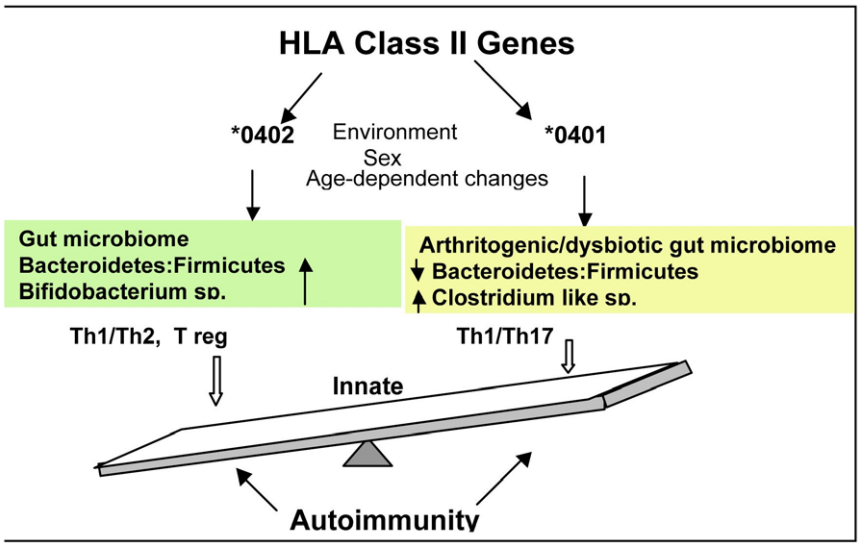

Figure 7. Role of the gut microbiome in susceptibility to arthritis. HLA genotype may shape the gut microbiome in an individual. The DRB1*0401 gene associated with predisposition to Rheumatoid arthritis, may induce a lower gut Bacteroidetes: Firmicutes ratio compared to that shaped by the DRB1*0402 gene, known to be associated with resistance to arthritis. This model suggests that a dysbiotic or arthritogenic gut microbiome may be dominated by a Clostridia-like bacterium (Firmicutes phylum) in susceptible individuals, while competent/tolerant immune responses are enhanced by increased abundances of *Bifidobacterium* spp. in resistance to RA. The gut microbiome has a crucial influence on maintaining homeostasis of the gut immune system by predicting pro-inflammatory (TH1/Th17) or anti-inflammatory (TH1/TH2) responses. Environmental triggers like smoking, diet and infectious agents along with sex hormones and age-dependent changes, may further modulate the gut immune system and enhance pro-inflammatory conditions in genetically susceptible individuals. In synthesis, an overall/systemic immune response generated by innate immune cells, may be originated at gut level and this response may be regulated by the gut microbiome via HLA genotype. This chain of events may determine the onset of autoimmune diseases like rheumatoid arthritis.
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www.ncbi.nlm.nih.gov/sra/), accession number SRA043819. NMDS plots and SIMPER analyses were constructed based on Bray-Curtis distance metrics using Primer-E from normalized OTU-abundance data. Correspondence analysis was plotted using the ca package and bipartite network analysis from the R project statistical software [53].

Induction and evaluation of CIA

To induce CIA, 8–12 weeks old *0401 transgenic mice and negative littermates were immunized with 100 µg of type II collagen (CII) (Chondrex Inc.) emulsified 1:1 with complete Freunds' adjuvant H37Ra (CFA, Difco Laboratories, Detroit, MI) intradermally at the base of the tail as previously described for CIA protocol [18]. Mice were monitored for the onset and progression of CIA from 3–12 weeks postimmunization. The arthritic severity of mice was evaluated as described previously with a grading system for each paw from 0–3 as described [20]. Mice with a score of 2 or more were used as arthritic mice.

Intestinal permeability

As gut permeability may be diet dependent, all transgenic mice were kept on standard diet. Changes in intestinal permeability were determined using 4-KDa FITC-labeled dextran. Mice were deprived of food for 3 hours, then gavaged with FITC–labeled dextran (0.6 mg/g body weight). Three hours later, mice were bled and serum collected. FITC-dextran content of the sera was determined by using a microplate reader with an excitation of 490 nm and emission detection at 525 nm. Gut permeability was tested in age and sex matched arthritic (7 weeks post-immunization with CII) and naïve (non-immunized) mice.

RNA isolation and Real- time Polymerase chain reaction

Jejenum of naïve transgenic mice were isolated from 4 months old mice. After a midline celiotomy, the intestine was flushed with cold (4°C) phosphate buffered saline (PBS) to remove intraluminal content and jejunal segments were placed in RNA stabilization buffer (Qiagen). Total RNA from the isolated tissue was extracted using the RNeasy kit and protocol (Qiagen). cDNA was prepared using RT2 First Strand Kit cDNA Synthesis Kit and Primer Mixes (SABiosciences). The quantification of gene expression related to the Th17 Regulatory Network was performed using the RT^2 Profiler PCR Array PAMM-0773 (SABiosciences) and the HT7900 Fast Real-Time PCR System (ABI). Product amplification was measured and analyzed according to the manufacturer's instructions.

Statistical analyses

NMMDS (Non-metric Multi-dimensional scale) plots, SIMPER (Percentages-species contribution) analyses and ANOSIM (Analysis of similarities) were constructed based on Bray-Curtis distance metrics using Primer-E (PRIMER 5, version 5.2.7 (Primer-E Ltd., Plymouth, United Kingdom. Clark, 2005) from square root transformed OTU-abundance data. The ANOSIM procedure generates a test statistic, R, calculated as: $R = (r_B - r_W)/[1/4n(n-1)]$, where n is the total number of samples, r_B is the average of rank similarities arising from all pairs of replicates between different mice fecal samples groups (Strain, sex or age), and r_W is defined as the average of all rank similarities among replicates within mice fecal samples groups. An R value of 1 indicates complete dissimilarity between groups; an R of 0 indicates a high degree of community similarity among groups.

Relative abundance of each OTU calculated as number of reads of a taxon/total number of reads in a sample was used to

construct correspondence analysis plots using the ca package (50) from the R project statistical software. To assess significant differences in relative abundances of specific OTUs between strain, sex or age groups non-parametric Mann-Whitney *U*/ Wilcoxon rank sum tests were conducted using the R project statistical tool. All data for the non-parametric tests were checked for homogeneity of error variances using the Brown-Forsythe test. Statistical significance was set to $P<0.05$. Non-parametric Spearman correlation coefficients were determined using the PROC COR procedure from the SAS software platform (SAS version 9.1.3; SAS Institute, Cary NC), appropriate significance level, error correction and power for all tests were determined using the pwr package from the R project statistical software. Significance difference in expression of gene transcripts for Th17 cytokines and regulating genes were analyzed by online tool available from the manufacturer for the PAMM0733 array (SABiosciences) and is reported as significant fold change difference of $p<0.05$ between groups. Difference in gut permeability between ages and sex was calculated by student's T test with significance set at $p<0.05$.

Supporting Information

Figure S1 Heat map showing expression levels of cytokines and chemokine transcripts in jejenum of *0401 and *0402 male and female mice.
(TIF)

Table S1 Correlation coefficients between OUT's and cytokine/chemokine transcript levels.
(DOCX)

File S1 Differences in Th17 regulatory network transcripts in *0401 and *0402 male and female mice.
(XML)

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Author Contributions

Conceived and designed the experiments: VT JAM BAW. Performed the experiments: AG DL EVM CJY MEB. Analyzed the data: AG CJY VT BAW. Contributed reagents/materials/analysis tools: VT BAW. Wrote the paper: AG VT CJY JAM BAW.

References

1. Klareskog L, Padyukov L, Lorentzen J, Alfredsson L (2006) Mechanisms of disease: Genetic susceptibility and environmental triggers in the development of rheumatoid arthritis. Nat Clin Pract Rheumatol 2: 425–433.
2. Moen K, Brun JG, Valen M, Skartveit L, Eribe EK, et al. (2006) Synovial inflammation in active rheumatoid arthritis and psoriatic arthritis facilitates trapping of a variety of oral bacterial DNAs. Clin Exp Rheumatol 24: 656–663.
3. Kempsell KE, Cox CJ, Hurle M, Wong A, Wilkie S, et al. (2000) Reverse transcriptase-PCR analysis of bacterial rRNA for detection and characterization of bacterial species in arthritis synovial tissue. Infect Immun 68: 6012–6026.
4. Round JL, O'Connell RM, Mazmanian SK (2010) Coordination of tolerogenic immune responses by the commensal microbiota. J Autoimmun 34: J220–225.
5. Chow J, Lee SM, Shen Y, Khosravi A, Mazmanian SK. Host-bacterial symbiosis in health and disease. Adv Immunol 107: 243–274.
6. Amirzargar A, Mytilineos J, Yousefipour A, Farjadian S, Scherer S, et al. (1998) HLA class II (DRB1, DQA1 and DQB1) associated genetic susceptibility in Iranian multiple sclerosis (MS) patients. Eur J Immunogenet 25: 297–301.
7. Vaahtovuo J, Munukka E, Korkeamaki M, Luukkainen R, Toivanen P (2008) Fecal microbiota in early rheumatoid arthritis. J Rheumatol 35: 1500–1505.
8. Finegold SMSV, Mathisen GE, In: editor. New York: Academic Press; 1983:3–31 (1983) Normal indigenous intestinal flora. In: Hentges DJ, ed. Human intestinal microflora in health and disease. New York: Academic Press. pp 3–31.
9. Langendijk PS, Schut F, Jansen GJ, Raangs GC, Kamphuis GR, et al. (1995) Quantitative fluorescence in situ hybridization of Bifidobacterium spp. with genus-specific 16S rRNA-targeted probes and its application in fecal samples. Appl Environ Microbiol 61: 3069–3075.
10. Ventura M, Turroni F, Canchaya C, Vaughan EE, O'Toole PW, et al. (2009) Microbial diversity in the human intestine and novel insights from metagenomics. Front Biosci 14: 3214–3221.
11. Liu YJ, Soumelis V, Watanabe N, Ito T, Wang YH, et al. (2007) TSLP: an epithelial cell cytokine that regulates T cell differentiation by conditioning dendritic cell maturation. Annu Rev Immunol 25: 193–219.
12. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A (2001) Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol 19: 683–765.
13. Saenz SA, Taylor BC, Artis D (2008) Welcome to the neighborhood: epithelial cell-derived cytokines license innate and adaptive immune responses at mucosal sites. Immunol Rev 226: 172–190.
14. Baba N, Samson S, Bourdet-Sicard R, Rubio M, Sarfati M (2008) Commensal bacteria trigger a full dendritic cell maturation program that promotes the expansion of non-Tr1 suppressor T cells. J Leukoc Biol 84: 468–476.
15. Christensen HR, Frokiaer H, Pestka JJ (2002) Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. J Immunol 168: 171–178.
16. Kelsall BL, Leon F (2005) Involvement of intestinal dendritic cells in oral tolerance, immunity to pathogens, and inflammatory bowel disease. Immunol Rev 206: 132–148.
17. Taneja V, Behrens M, Basal E, Sparks J, Griffiths MM, et al. (2008) Delineating the role of the HLA-DR4 "shared epitope" in susceptibility versus resistance to develop arthritis. J Immunol 181: 2869–2877.
18. Taneja V, Behrens M, Mangalam A, Griffiths MM, Luthra HS, et al. (2007) New humanized HLA-DR4-transgenic mice that mimic the sex bias of rheumatoid arthritis. Arthritis Rheum 56: 69–78.
19. Geluk A, Taneja V, van Meijgaarden KE, Zanelli E, Abou-Zeid C, et al. (1998) Identification of HLA class II-restricted determinants of Mycobacterium tuberculosis-derived proteins by using HLA-transgenic, class II-deficient mice. Proc Natl Acad Sci U S A 95: 10797–10802.
20. Behrens M, Trejo T, Luthra H, Griffiths M, David CS, et al. (2010) Mechanism by which HLA-DR4 regulates sex-bias of arthritis in humanized mice. J Autoimmun.
21. Vaahtovuo J, Toivanen P, Eerola E (2003) Bacterial composition of murine fecal microflora is indigenous and genetically guided. FEMS Microbiol Ecol 44: 131–136.
22. De Palma G, Capilla A, Nadal I, Nova E, Pozo T, et al. (2009) Interplay Between Human Leukocyte Antigen Genes and the Microbial Colonization Process of the Newborn Intestine. Curr Issues Mol Biol 12: 1–10.
23. Mariat D, Firmesse O, Levenez F, Guimaraes V, Sokol H, et al. (2009) The Firmicutes/Bacteroidetes ratio of the human microbiota changes with age. BMC Microbiol 9: 123.
24. Mueller S, Saunier K, Hanisch C, Norin E, Alm L, et al. (2006) Differences in fecal microbiota in different European study populations in relation to age, gender, and country: a cross-sectional study. Appl Environ Microbiol 72: 1027–1033.
25. Kovacs A, Ben-Jacob N, Tayem H, Halperin E, Iraqi FA, et al. (2011) Genotype is a stronger determinant than sex of the mouse gut microbiota. Microb Ecol 61: 423–428.
26. Chervonsky AV (2010) Influence of microbial environment on autoimmunity. Nat Immunol 11: 28–35.
27. Lee YK, Mazmanian SK (2010) Has the microbiota played a critical role in the evolution of the adaptive immune system? Science 330: 1768–1773.
28. Ley RE, Peterson DA, Gordon JI (2006) Ecological and evolutionary forces shaping microbial diversity in the human intestine. Cell 124: 837–848.
29. Taneja V, Taneja N, Behrens M, Pan S, Trejo T, et al. (2003) HLA-DRB1*0402 (DW10) transgene protects collagen-induced arthritis-susceptible H2Aq and DRB1*0401 (DW4) transgenic mice from arthritis. J Immunol 171: 4431–4438.
30. Wen L, Ley RE, Volchkov PY, Stranges PB, Avanesyan L, et al. (2008) Innate immunity and intestinal microbiota in the development of Type 1 diabetes. Nature 455: 1109–1113.
31. Ichinohe T, Pang IK, Kumamoto Y, Peaper DR, Ho JH, et al. (2011) Microbiota regulates immune defense against respiratory tract influenza A virus infection. Proc Natl Acad Sci U S A 108: 5354–5359.
32. Zaph C (2010) Which species are in your feces? J Clin Invest 120: 4182–4185.
33. Ito T, Carson WFt, Cavassani KA, Connett JM, Kunkel SL. CCR6 as a mediator of immunity in the lung and gut. Exp Cell Res 317: 613–619.
34. Lugering A, Floer M, Westphal S, Maaser C, Spahn TW, et al. (2005) Absence of CCR6 inhibits CD4+ regulatory T-cell development and M-cell formation inside Peyer's patches. Am J Pathol 166: 1647–1654.

35. McDonald KG, Leach MR, Huang C, Wang C, Newberry RD. Aging impacts isolated lymphoid follicle development and function. Immun Ageing 8: 1.
36. Mazmanian SK, Round JL, Kasper DL (2008) A microbial symbiosis factor prevents intestinal inflammatory disease. Nature 453: 620–625.
37. Matsumoto M, Kurihara S, Kibe R, Ashida H, Benno Y (2011) Longevity in mice is promoted by probiotic-induced suppression of colonic senescence dependent on upregulation of gut bacterial polyamine production. PLoS One 6: e23652.
38. Cerf-Bensussan N, Gaboriau-Routhiau V (2010) The immune system and the gut microbiota: friends or foes? Nat Rev Immunol 10: 735–744.
39. Kverka M, Zakostelska Z, Klimesova K, Sokol D, Hudcovic T, et al. (2011) Oral administration of Parabacteroides distasonis antigens attenuates experimental murine colitis through modulation of immunity and microbiota composition. Clin Exp Immunol 163: 250–259.
40. Lathrop SK, Bloom SM, Rao SM, Nutsch K, Lio CW, et al. (2011) Peripheral education of the immune system by colonic commensal microbiota. Nature 478: 250–254.
41. Caselli M, Holton J, Boldrini P, Vaira D, Calo G (2010) Morphology of segmented filamentous bacteria and their patterns of contact with the follicle-associated epithelium of the mouse terminal ileum: implications for the relationship with the immune system. Gut Microbes 1: 367–372.
42. Fuentes S, Egert M, Jimenez-Valera M, Monteoliva-Sanchez M, Ruiz-Bravo A, et al. (2008) A strain of Lactobacillus plantarum affects segmented filamentous bacteria in the intestine of immunosuppressed mice. FEMS Microbiol Ecol 63: 65–72.
43. Greetham HL, Gibson GR, Giffard C, Hippe H, Merkhoffer B, et al. (2004) Allobaculum stercoricanis gen. nov., sp. nov., isolated from canine feces. Anaerobe 10: 301–307.
44. Mangin I, Bonnet R, Seksik P, Rigottier-Gois L, Sutren M, et al. (2004) Molecular inventory of faecal microflora in patients with Crohn's disease. FEMS Microbiol Ecol 50: 25–36.
45. Benno Y, Mitsuoka T (1992) Impact of Bifidobacterium longum on human fecal microflora. Microbiol Immunol 36: 683–694.
46. Sait LC, Galic M, Price JD, Simpfendorfer KR, Diavatopoulos DA, et al. (2007) Secretory antibodies reduce systemic antibody responses against the gastrointestinal commensal flora. Int Immunol 19: 257–265.
47. Alauzet C, Mory F, Carlier JP, Marchandin H, Jumas-Bilak E, et al. (2007) Prevotella nanceiensis sp. nov., isolated from human clinical samples. Int J Syst Evol Microbiol 57: 2216–2220.
48. Picco P, Gattorno M, Marchese N, Vignola S, Sormani MP, et al. (2000) Increased gut permeability in juvenile chronic arthritides. A multivariate analysis of the diagnostic parameters. Clin Exp Rheumatol 18: 773–778.
49. Ng SC, Hart AL, Kamm MA, Stagg AJ, Knight SC (2009) Mechanisms of action of probiotics: recent advances. Inflamm Bowel Dis 15: 300–310.
50. So JS, Kwon HK, Lee CG, Yi HJ, Park JA, et al. (2008) Lactobacillus casei suppresses experimental arthritis by down-regulating T helper 1 effector functions. Mol Immunol 45: 2690–2699.
51. Kouskoff V, Fehling HJ, Lemeur M, Benoist C, Mathis D (1993) A vector driving the expression of foreign cDNAs in the MHC class II-positive cells of transgenic mice. J Immunol Methods 166: 287–291.
52. Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, et al. (2009) Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol 75: 7537–7541.
53. Nenadic O, Greenacre M (2007) Correspondence analysis in R, with two and three dimensional Graphics: the ca package. J Statis Soft 20: 1–13.